

USE IT AND BOOST IT: COGNITIVE AND PHYSICAL ACTIVITIES MODULATE
COGNITION VIA BDNF-TRKB SIGNALING

BY

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DISSERTATION

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ABSTRACT

Our motto “Use it and boost it” suggests that stimulating experiences enhance neural functioning. Previous work has shown that cognition is enhanced by mental and physical activities. The current research examines the molecular mechanisms underlying cognitive and physical activity-induced enhancements in cognition. We found that cognitive and physical activity increased BDNF protein, BDNF-TrkB signaling, and BDNF release in the hippocampus and striatum. Our studies show that priming the brain with cognitive and physical activities enhance hippocampus- and striatum-sensitive learning via BDNF-TrkB signaling. In addition, we found that physical activity increases BDNF release in the striatum before and during task learning and in the hippocampus during task learning. To further identify the cell signaling mechanisms supporting cognitive activity-induced learning enhancements, we investigated how BDNF-TrkB signaling and learning contribute to GSK3 β inhibition in the hippocampus and striatum. We found that GSK3 β inhibition in the hippocampus and striatum increased following place and response training, but not the priming task SA. Furthermore, we found no effects of blockade of BDNF-TrkB signaling on GSK3 β inhibition in the hippocampus and striatum. Thus, BDNF-TrkB signaling induced by cognitive priming is modulating place and response learning enhancements via non GSK3 β -related mechanisms. Taken together, our results show that the molecular mechanisms underlying our motto of “Use it and Boost it” are functioning via use-induced increases in BDNF release, and signaling in the hippocampus and striatum.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Learning how to learn

“If you can change your mind, you can change your life” –William James

In 1890, William James originated the idea of “metacognitive experiential learning”.

This theory focused on learning from experience. Our lives are spent going from experience to experience, learning new information along the way, and with each new experience we behave differently than we would have had we not had the prior experiences.

Thus, experience primes future experience, but how, at a cellular level? Is it something specific about each experience that prepares us for future experiences? Or is it just engaging in the experience in itself that allows for changes in our brain and thus our behavior? Throughout this dissertation I will be unable to directly ask or answer these questions; however, I will attempt to offer further support of his theory via analyses of cellular adaptations in neural systems that take part in learning. I hypothesize that priming the brain with cognitive and physical activity can up-regulate BDNF release and signaling to support the functioning of neural systems (i.e. Use it and boost it). Our “Use it and Boost it” theory builds on James’ idea of metacognitive experiential learning: learning new information improves future learning due to increases in BDNF signaling in primed neural systems.

Boosting and testing multiple memory systems

“There is more than one kind of learning” (Tolman, 1949) and multiple memory systems support different learned behaviors. In the current research, food motivated T-mazes measure learning performance in the hippocampus-sensitive place task and the striatum-sensitive response task. In rodents, the contributions of the hippocampus to place learning and striatum to response learning have been demonstrated by lesions (McDonald and White, 1993; Chang and Gold 2003b; Chang and Gold 2004), neurochemical shifts (Chang and Gold 2003a; Chapter 4; Gold et al., 2011; Gold et al., 2013; Korol, 2004; Pych et al., 2005a; 2005b), and activity-dependent cell signaling cascades (Colombo et al., 2003; Pisani et al., 2012). There is a plethora of evidence supporting the hippocampus and striatum as competing memory systems (for review see Poldrack and Packard, 2003). For example, lesion studies have found that inactivation of the hippocampus not only impairs place learning but also enhances response learning and vice versa for inactivation of the striatum (McDonald and White, 1993; Chang and Gold 2003a; 2004). Extracellular acetylcholine, glucose, lactate, and BDNF increase in the hippocampus compared to the striatum when a rat engages in place strategies and in the striatum compared to the hippocampus when a rat engages in response strategies (Gold et al., 2011; Newman et al., in prep; Pych et al., 2005a; Chapter 4). On the other hand, studies of estrogen effects on learning and memory point to independent functions of the hippocampus and striatum to regulate place and response learning (Zurkovsky et al., 2007). Alternatively, electrophysiological studies point to collaborative functions of the hippocampus and striatum during place and response learning. Both the hippocampus and striatum increase neural activity during

place and response tasks. Furthermore, both the hippocampus and striatum increase neural activity in response to both movement and spatial cues (Yeshenko et al., 2004).

The current research provides support for the hippocampus and striatum as competing, independent, and collaborative memory systems. We report evidence of the hippocampus and striatum working independently, with cognitive and physical activity enhancing both place and response learning in the absence of apparent competition. In addition, cognitive and physical activities increase BDNF protein content, signaling, and release in both the hippocampus and striatum (Korol et al., 2013; Chapters 2-4).

However, we have neurochemical data supporting competition between the hippocampus and striatum. Specifically, BDNF release patterns are significantly different in the hippocampus and striatum during place and response learning. BDNF release in the hippocampus is higher during place learning compared to response learning and higher in the striatum during response learning compared to place learning (Chapter 4). In addition, we have evidence of the hippocampus and striatum working collaboratively. In Chapter 5, we found that following place and response training, the activity-dependent inhibition of glycogen synthase kinase 3 β (GSK3 β) was increased in both the hippocampus and striatum.

Cognitive and physical activity globally enhance learning and memory

Across a variety of learning and memory tasks, physically active animals excel compared to sedentary controls (Chapter 2, Erickson et al., 2011; van Praag et al., 2005; Korol et al., 2013; Colcombe and Kramer, 2003). And the cognitive benefits of physical activity continue throughout old age (for review see Churchill et al., 2002). Like physical activity, engaging in cognitive activity early in life supports learning and

memory enhancements across the lifespan. In Snowden's Nun study, a study of 687 elderly nuns, those with higher linguistic ability early in life were less likely to suffer from cognitive decline during aging, and less likely to have neuropathological hallmarks of Alzheimer's disease (Riley et al., 2005).

Cognitive training of certain tasks enhances future behaviors that rely on previously practiced domains of cognition (Harlow, 1949; Ericsson & Chase, 1982; Kleinknecht et al., 2012; Ball et al., 2002). For example, rats trained in a complex spatial environment acquired the spatial version of the swim task faster than controls (Moser et al., 1994). Like physical activity, the effects of cognitive training on cognition are not age-limited; cognitive training during old age also enhances cognition in the elderly. Elderly adults engaged in strategy training for memory, reasoning, or speed of processing skills showed reliable enhancements in cognitive tests specific to the trained skill set for up to 2 years following training (Ball et al., 2002). On the other hand, cognitive training via working memory testing is beneficial for a variety of tasks that tap multiple domains of cognition (Chien and Morrison, 2010; Jaeggi et al., 2008; Chapter 3; Light et al., 2010; Matzel et al., 2011; Korol et al., 2013).

Environmental enrichment (EE), which is a stimulating environment full of novel objects, exercise wheels, and social interactions, globally enhances cognition (Greenough et al., 1969; 1972; Krech et al., 1962; van Praag et al., 2000; Leggio et al., 2005) and globally changes the chemistry and structure of the brain (Bennett et al., 1964; Landers et al., 2011; Mychasiuk et al., 2014; Mora et al., 2007; Diamond et al., 1967; 1964; Globus et al., 1973; Krech et al., 1960; Leggio et al., 2005). EE, containing both cognitive and physical activity, is a perfect example of the "Use it and Boost it"

theory in action. For example, priming with EE enhances spatial learning (Leggio et al., 2005; Toscano et al., 2006), working memory (Hoplight et al., 2001), object recognition memory (Tees, 1999), and motor learning (Lonetti et al., 2010). In addition, the neurobiological data from EE animals report increased neurotrophic factors—specifically brain-derived neurotrophic factor (BDNF) and fibroblast growth factor (FGF) in the hippocampus and cortex (Ickes et al., 2000; Cao et al., 2004), increased angiogenesis (Black et al., 1987; Sirevaag et al., 1988; Cao et al., 2004), increased hippocampal neurogenesis (Kempermann et al., 1998), and increased dendritic arborization and spine density (Leggio et al., 2005; Volkmar et al., 1972; Moser et al., 1994). Interestingly, all the EE-induced changes in the brain are also observed following physical activity, cognitive activity, or both (Gomez-Pinilla et al., 1997; van Praag et al., 1999; Neeper et al., 1995; Klintsova et al., 2004; Cotman and Berchtold, 2002; Eadie et al., 2005; Isaacs et al., 1992; Black et al., 1990; Mizuno et al., 2000; Berchtold et al., 2005; Korol et al., 2013; Chapters 2 and 3).

Although studies have attempted to separate the roles of cognitive and physical activity on the structural changes that occur due to EE (Black et al., 1990; Issacs et al., 1992; Anderson et al., 1994; Lambert et al., 2005), it has been difficult to identify one or both components of EE (cognitive or physical activity) as an underlying cause of EE-induced learning enhancements. For example, EE increases synapse formation and capillary density due to cognitive and physical activity, respectively (Black et al., 1990; Moser et al., 1994). Importantly, BDNF-TrkB signaling is an important modulator of both synaptogenesis (Haraguchi et al., 2012) and angiogenesis (Kermani and Hempstead, 2007).

Evidence from our studies suggests that cognitive and physical activity enhance learning via a reliance on BDNF-TrkB signaling (Korol et al., 2013; Chapters 2 and 3). In Korol et al., 2013 (data presented in Chapters 2 and 3), both cognitive and physical activity independently enhanced place and response learning. Hippocampus and striatum infusions of the TrkB inhibitor, K252a, attenuated cognitive and physical activity-induced enhancements in place and response learning. In Chapter 4, we report that physical activity increased BDNF release in the hippocampus during place learning and in the striatum before and during response learning. BDNF release in the hippocampus and striatum was also increased due to the cognitive priming task, spontaneous alternation (SA), which we used for our studies in Chapter 3 (unpublished data, Figure 1.1). Taken together, our findings from Chapters 2-4 show that both cognitive and physical activity enhance place and response learning and increase BDNF protein content, signaling, and release in the hippocampus and striatum.

Cognitive and physical activity stimulate BDNF-TrkB signaling, which supports future cognitive activity

BDNF has long been acknowledged as an activity-dependent protein, with its gene expression and release modulated specifically by learning and/or increases in neural activity (Chapter 4; Kuczewski et al., 2008; Aicardi et al., 2004; Zafra et al., 1990; Kesslak et al., 1998; Castren et al., 1992). For example, physiological and electrophysiological stimulation of neurons *in vitro* results in increases in BDNF mRNA transcripts and extracellular mBDNF levels, indicating activity-induced increases in secretion, expression, and signaling of mBDNF (Lu, 2003; Schwartz et al., 2011; Kuczewski et al., 2008; Zafra et al., 1990; Balkowiec and Katz, 2000; Hartmann et al.,

2001). Notably, engaging in cognitive and physical activities induces rapid and durable increases in mature BDNF (mBDNF) protein and signaling in the brain (Chapter 2 and 3; Kim et al., 2012; Berchtold et al., 2005; Hall et al., 2000).

BDNF release is both constitutive and regulated (for review see, Lessman and Brigadski, 2009). Constitutive release of BDNF is unaffected by increases in neuronal activity, while the regulated release of mBDNF is activity-dependent (Hartmann et al., 2001; Canossa et al., 1997; Goodman et al., 1996). The activity-dependent release of BDNF is especially important for learning and memory, and for the beneficial effects of physical activity on learning and memory (Erickson et al., 2013; Egan et al., 2003). For example, a single nucleotide polymorphism in the BDNF gene (Val66Met) results in an inability to sort BDNF into the regulated release pathway (Chen et al., 2004). Thus, Val66Met individuals have learning and memory deficits, are immune to the cognitive-enhancing effects of exercise, and are more likely to suffer from neurodegenerative disease (Erickson et al., 2013; Egan et al., 2003; Lim et al., 2014; Ventriglia et al., 2002; Hariri et al., 2003).

In this dissertation we—in the first experiments ever to do so— demonstrated the cognitive and physical activity-dependence of BDNF. In Chapter 4, we developed a method to measure BDNF release *in vivo* in awake, behaving animals. We found that the cognitive and physical activity-induced increases in BDNF release are specific to neural sites involved in the activity (see Chapter 4). Other studies have corroborating evidence of the cognitive and physical activity dependence of BDNF release and signaling. For example, BDNF mRNA and protein levels increase in the hippocampus and striatum immediately following learning (Kesslak et al., 1998, Kim et al., 2012; Hall

et al., 2000; Mizuno et al., 2000; Chapter 3; Korol et al., 2013; Figure 1.2). During exhaustive exercise, and following endurance training, plasma levels of brain-derived BDNF increase in the periphery (Rasmussen et al., 2009; Seifert et al., 2010). The hippocampus increases BDNF mRNA following physical activity (Neeper et al., 1995; Neeper et al., 1996) and both the hippocampus and striatum have increased mBDNF protein concentrations following both physical and cognitive activity (Chapters 2 and 3; Kim et al., 2012; Hall et al., 2000; Ding et al., 2004b; Korol et al., 2013). Furthermore, cognitive and physical activity-induced increases in BDNF protein persist for several hours following the activity (Berchtold et al., 2005; Kim et al., 2012; Chapter 3). Given that increases in neural activity lead to increases in BDNF protein and proteins downstream of the BDNF-TrkB signaling pathway (Molteni et al., 2002), it is likely that activity-induced increases in BDNF support future neural activity. The studies presented in this dissertation report that priming the brain with cognitive and physical activity increases a reliance on BDNF-TrkB signaling during probe tasks, and that this increased reliance on BDNF-TrkB signaling is a modulator of activity-induced learning enhancements (Korol et al., 2013; Chapters 2 and 3). In addition, we found that BDNF release in the hippocampus is higher in physically active rats that were the best place learners (Chapter 4). The data from this dissertation support a role for the rapid, not trophic, effects of BDNF-TrkB signaling during learning in activity-induced learning enhancements.

Rapid effects of BDNF-TrkB signaling during and following increases in neural activity

Many of the rapid signaling cascades downstream of BDNF-TrkB are associated with enhanced learning and memory processes and neural plasticity. For example, rapid signaling events associated with BDNF-TrkB lead to increased AMPA receptor concentrations on the post-synaptic membrane (Du and Poo, 2004), intracellular calcium (He et al., 2005; Fenner et al., 2012; Du and Poo, 2004), ERK activation (Ji et al., 2010; Johnson-Farley et al., 2007), neurotransmitter release (Lessman and Heumann 1998; Knipper et al., 1994; Sala et al., 1998; Santafe et al., 2014; Tyler and Pozzo-Miller, 2001; Numakawa et al., 1998; Du and Poo, 2004), dendritic protein translation (Schratt et al., 2004; Yin et al., 2002; for review see Bramham and Messoudi, 2005), and BDNF release (Bramham and Messoudi, 2005; Canossa et al., 1997). In addition, BDNF- TrkB activates PI3K/Akt (Yamada et al., 1997; Yamada et al., 2001), leading to increased glucose use (Burkhalter et al., 2003) and inactivation of GSK3 β (Li et al., 2007; Hoppe et al., 2013). Thus, activity-induced increases in BDNF protein and signaling likely support and initiate molecular changes that prime the neural systems that were in “use” for a “boost” when engaged in the future.

Pharmacological activation of the TrkB signaling cascade prior to task acquisition enhances memory (Andero et al., 2011). The timing of agonist administration (1 hr prior to learning) suggests that acute signaling and not trophic effects of TrkB receptor activation mediate memory enhancements. Careful analysis of the TrkB signaling pathway could point to one, some, or all the different rapid signaling cascades downstream of TrkB activation (i.e. increased intracellular [Ca⁺⁺], ERK activation, increased energy availability, and/or inhibition of GSK3 β). There is a large literature implicating GSK3 β hyperactivity as an indicator of decreased cell health and impaired

learning and memory (Hernandez et al., 2002; for review see Johe and Johnson 2004; Hooper et al., 2007a; 2007b). It is our hypothesis that BDNF-TrkB signaling mediates the effects of activity-induced learning enhancements by inhibiting GSK3 β .

GSK3 β , originally discovered as a regulator of glycogen synthesis, is a constitutively active protein kinase with almost 100 substrates including tau and β -catenin (Sutherland et al., 2011). Inactivation of GSK3 β , which is beneficial for learning and memory processes (Hooper, 2007; Peineau, 2007), stimulates glycogen synthesis in astrocytes (Heni et al., 2011; Sanchez et al., 1998) and neuroprotection and neuroplasticity mechanisms in neurons (Chin et al., 2005; Peineau et al., 2007). On the contrary, hyperactivity of GSK3 β results in learning and memory deficits (Lucas et al., 2001; Wagner et al., 1996; Rubinfeld et al., 1996, Hernandez et al., 2002) and studies have found that pharmacological inhibition of GSK3 β in animals with Alzheimer's-like deficits does indeed improve memory (Sereno et al., 2009; Ponce-Lopez et al., 2011).

Given that BDNF protein content, release, and signaling increase following cognitive and physical activity, it is likely that GSK3 β inhibition is occurring as well. I hypothesized that GSK3 β inhibition is a possible target in the BDNF-TrkB signaling pathway by which cognitive and physical activities are able to enhance future learning. Others have found that long-term treadmill exercise increases GSK3 β inhibition in the hippocampus (Bayod et al., 2011; 2014). Our results (described in Chapter 5) found that place and response learning, but not SA testing, increased GSK3 β inhibition in the hippocampus and striatum. Given that BDNF is increased in the hippocampus and striatum following SA testing, and the mechanism by which priming with SA testing enhances place and response learning is via BDNF signaling (Chapter 3) it was

surprising to see no effect of SA testing on GSK3 β inhibition. However, there was no effect of BDNF-TrkB signaling on GSK3 β inhibition in the hippocampus or striatum of place or response trained rats. Thus, other signaling cascades downstream of BDNF-TrkB signaling are likely modulating the effects of cognitive priming on place and response learning.

BDNF-TrkB signaling modulates many cellular activities, in both neurons and glia. Although neurons and glia both respond to BDNF via TrkB receptors, and induce similar intracellular cascades, they do so via different mechanisms. Neurons predominantly express full-length TrkB receptors, which rely on mBDNF binding to the receptor, leading to dimerization, and tyrosine kinase-mediated autophosphorylation of the receptor complexes. Autophosphorylation of TrkB receptor initiates downstream cascades within the neuron. However, glial cells express truncated TrkB receptors (Biffo et al., 1995; Klein et al., 1990), which do not have phosphorylation sites and thus do not dimerize. Instead in glia, a conformational change in the truncated TrkB receptor upon mBDNF binding activates a G-protein (G_q), leading to PLC γ activation, which increases intracellular calcium concentrations ($[Ca^{2+}]$) (Rose et al., 2013; for review see, Fenner, 2012). In glia, increases in intracellular $[Ca^{2+}]$ initiate many of the TrkB signaling cascades present in neurons (Figure 1.3). The tyrosine kinase inhibitor, K252a, used in our studies (Chapters 2, 3, and 5) acts on the full-length version of the TrkB receptor (i.e. the neuronal subtype). Thus, the effects on cognition induced by K252a treatment in our experiments will mainly affect neuronal TrkB signaling due to the inhibition of tyrosine kinase activity on the full-length TrkB receptor. Given that we do see behavioral changes due to K252a treatment in rats primed with cognitive and physical

activity (Chapters 2 and 3), we can link the priming effects of BDNF-TrkB signaling on probe tasks through neuronal, and not glial, BDNF-TrkB signaling. It is possible that the cognitively primed rats treated with K252a did not show decreases in GSK3 β inhibition in the hippocampus and striatum due to GSK3 β inhibition in the glia (that would be unaffected by K252a treatment). Perhaps, maintained GSK3 β inhibition in glia, overshadowed neuronal GSK3 β inhibition in primed K252a-treated rats in Chapter 5.

The mechanism of BDNF release differs across neural systems, in particular the hippocampus and striatum. In cultured hippocampal neurons BDNF releases into the extracellular space from activated neurons, both pre and post synaptically (Hartmann et al., 2001; Balkowiec and Katz, 2002; for review see Edelman et al., 2014). Thus, BDNF-TrkB signaling in the hippocampus is magnified, happening both pre- and post-synaptically. This reciprocal release and signaling of BDNF by communicating neurons is hypothesized to potentiate the synaptic signal (Bramham and Messoudi, 2005). However, in cultured striatal neurons, BDNF release occurs only from the presynaptic neuron (Jia et al., 2010); thus BDNF-TrkB signaling in the striatum occurs anterogradely, acting on the postsynaptic neuron. Currently it seems unclear why the hippocampus and striatum engage in different mechanisms of BDNF release or if these differences have relevance in mediating the effects of BDNF on learning. These data, however, do support our findings of larger quantities of BDNF protein in tissue homogenate and extracellular fluid in the hippocampus compared to the striatum (Chapter 3 and Chapter 4).

Summary

BDNF-TrkB signaling has a demonstrable role in learning and memory as well as long-term potentiation (LTP), a now accepted cellular model of learning and memory, in both the hippocampus and striatum (Lu, 2003; Gottschalk et al., 1999; Jia et al., 2010 Korte, et al., 1995; Korte et al., 1998; Linarrson et al., 1997; Mu et al., 1999). Despite the aforementioned differences in BDNF-TrkB signaling across neural systems and cell types, it is a goal in my dissertation to uncover the role of BDNF-TrkB signaling in the hippocampus and striatum in cognitive and physical activity-induced learning enhancements. Given that BDNF is an activity-dependent protein it is likely that the signaling mediated by BDNF-TrkB during and following activity is the mechanism supporting cognitive and physical activity-induced learning enhancements.

I argue that increases in BDNF tissue content, release, and signaling, observed during and immediately following cognitive or physical activity prime the brain for future learning. In the following studies, I explored the role of BDNF signaling underlying learning enhancements induced by cognitive and physical activity. In these investigations I used methods to measure place and response learning; hippocampal and striatal BDNF content, release, signaling; and inhibition of GSK3 β .

The “use it and boost it” theory proposes that regularly engaged neural systems induce adaptations that enhance future functioning. Thus, engaging in an experience provides us with the molecular machinery to support better learning in the future. As discussed above, increased BDNF signaling enhances learning and memory (Chapters 2, 3 and 4; Korol et al., 2013; Andero et al., 2011; D’Amore et al., 2013). Thus, it is likely that increases in BDNF-TrkB signaling, due to frequent use of neural systems (i.e. prior

experience), are able to bolster the future functioning of often used neural systems.

The following sets of studies offer a neurotrophic mechanism to support the theory of “use it and boost it”.

FIGURES

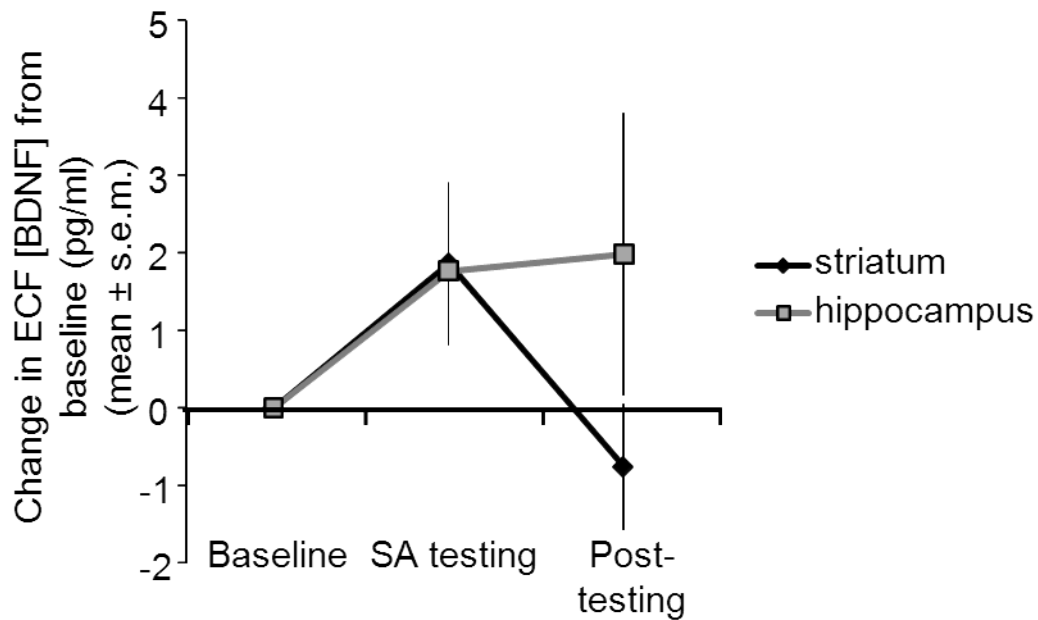


Figure 1.1 BDNF release in the hippocampus and striatum during spontaneous alternation testing. BDNF release increases in the hippocampus and striatum during SA-testing. Following SA testing, ECF BDNF levels in the hippocampus remain elevated, while the striatum decreases back down to baseline. N=3 for striatum and hippocampus.

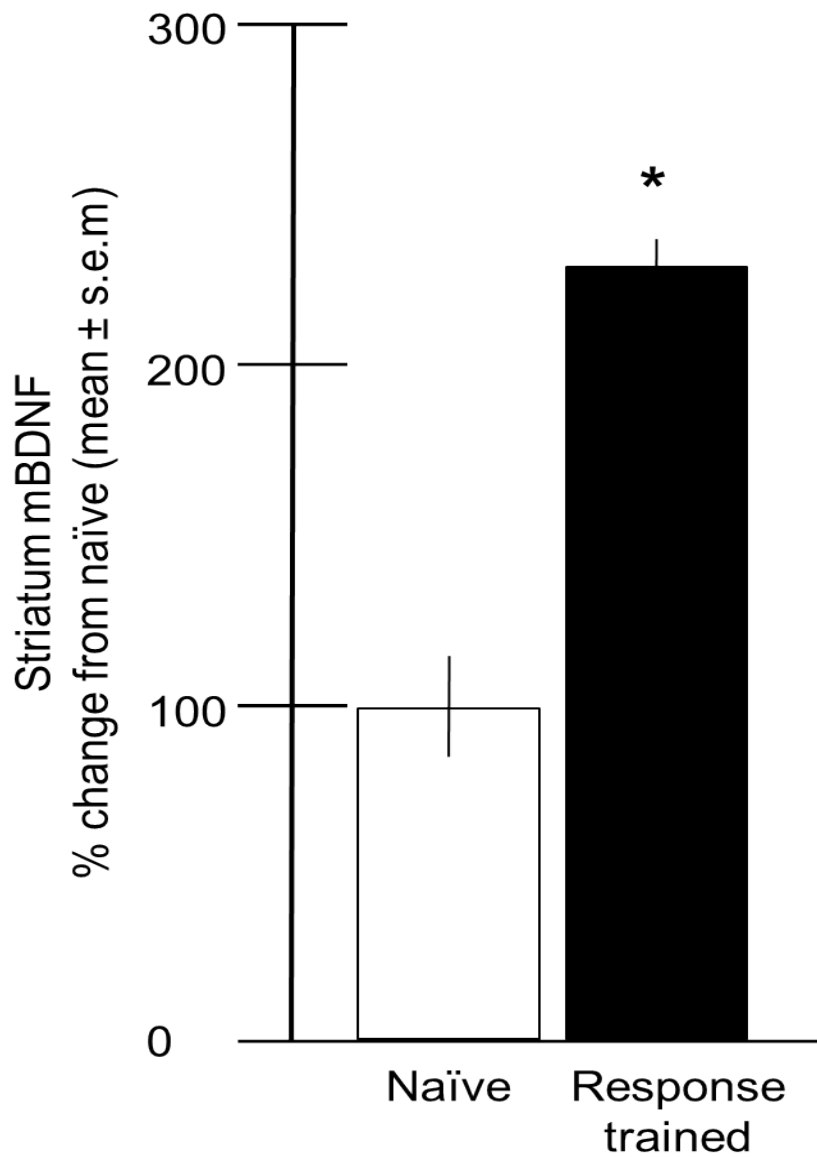


Figure 1.2 mBDNF tissue content in the striatum after response training.

Following response training, mBDNF concentrations in the striatum significantly increased compared to naïve controls. *= $p < 0.05$. N=8 for Naïve; N=9 for response trained.

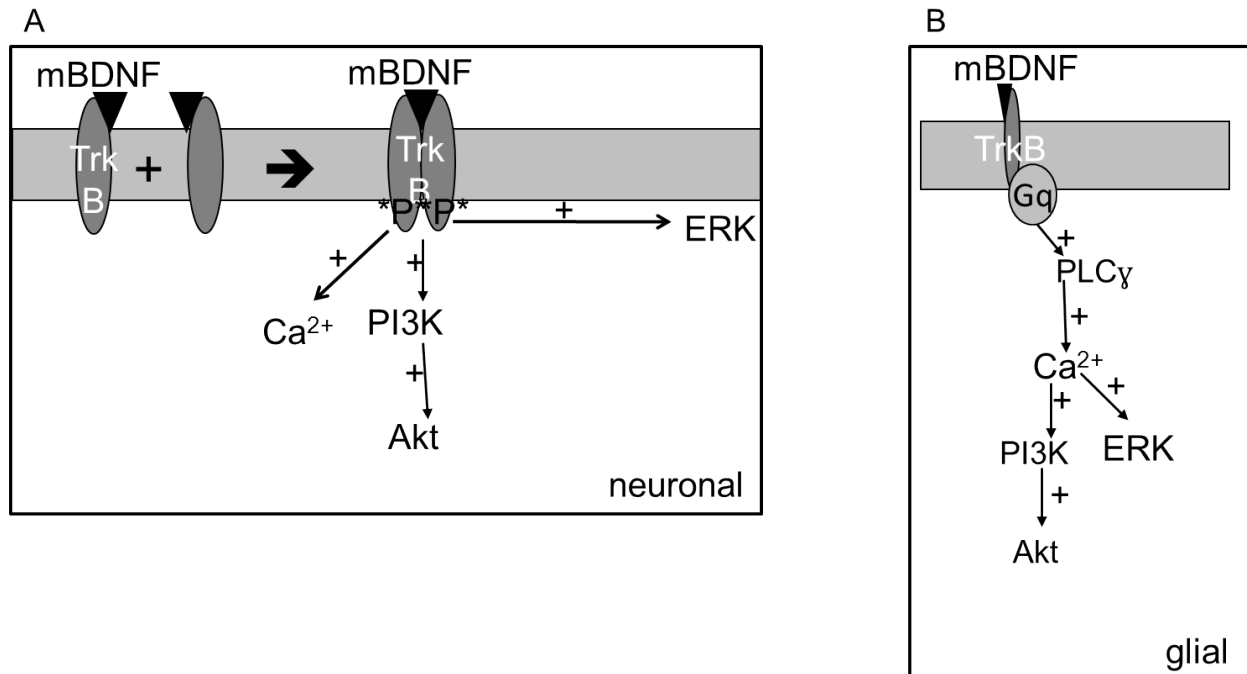


Figure 1.3 BDNF-TrkB signaling in the neuron versus the astrocyte. TrkB receptors are found on both A) the neuron and B) the astrocyte. Signaling in A) the neuron is initiated via mBDNF binding to the TrkB receptor, and subsequent dimerization of the ligand-receptor complexes. The dimerized BDNF-TrkB receptor complex activates tyrosine kinases on the intracellular domain of the receptors leading to receptor autophosphorylation, thus initiating signaling cascades (ERK, increases in intracellular $[Ca^{2+}]$, and PI3K/Akt). On the B) astrocyte, the TrkB receptor is truncated and lacks an intracellular domain. Thus upon mBDNF binding to the receptor, no dimerization or autophosphorylation takes place. Instead, ligand binding initiates a morphological change in the receptor causing the activation of a G protein (G_q), which activates PLC γ , leading to an increase in intracellular $[Ca^{2+}]$. Increases in intracellular $[Ca^{2+}]$ allow for the activation of TrkB signaling cascades such as PI3K/Akt and ERK.

CHAPTER 2: BDNF SIGNALING DURING LEARNING MODULATES EXERCISE-INDUCED LEARNING ENHANCEMENTS

ABSTRACT

Regular physical activity increases brain-derived neurotrophic factor (BDNF) in the hippocampus and striatum. Given the reported role of BDNF as an important modulator of learning and memory processes, we examined the effects of three weeks of voluntary exercise on striatum-sensitive response learning and hippocampus-sensitive place learning, and whether changes in mature BDNF (mBDNF) signaling mediated these effects. Three-month-old male Sprague-Dawley rats were exposed to voluntary wheel running or sedentary conditions. In the first experiment, physical activity enhanced learning in both place and response tasks and increased protein levels of mBDNF in the striatum and frontal cortex. The second experiment tested the role of BDNF-TrkB signaling in the learning enhancements observed after voluntary wheel running. We infused K252a, an inhibitor of TrkB signaling, or vehicle into the hippocampus 30 min before training on a place task or into the striatum 30 min before training on a response task. Infusions of K252a attenuated the exercise-induced place and response learning enhancements. Importantly, K252a treatment in sedentary rats failed to impair learning. These data extend findings of exercise-induced cognitive enhancements to striatum-based tasks and point to the importance of BDNF-TrkB signaling during learning as a candidate mechanism for exercise-induced cognitive enhancements. Our results suggest that rapid downstream consequences of mBDNF signaling are likely mediators of exercise-induced learning enhancements.

INTRODUCTION

Many studies demonstrate that the brain in particular benefits from physical activity, and by extension so do learning and memory processes. However, only certain aspects of cognition have been heavily studied, most often involving tasks that rely on intact hippocampal and frontal cortical functioning. For example, physically active rodents and humans show significant enhancements in spatial learning and executive function tasks compared to sedentary or relatively less fit controls (Anderson et al., 2000; Griffin et al., 2009; 2011; Colcombe and Kramer, 2003).

Because the neurobiological consequences of physical activity span many processes, from increased angiogenesis, to potentiated neurotransmission, to activation of molecular pathways, there is no obvious or single mechanism identified to underlie exercise-induced improvements in cognitive ability. However, through many different approaches, neurotrophic factors such as BDNF, have become widely accepted components of molecular cascades mediating physical activity-induced cognitive enhancements (for reviews see Hennigan et al. 2007; Cotman and Berchtold, 2002; Gomez-Pinilla, 2008; Kramer et al. 2006; Lojovich, 2010; Beckinschtein et al., 2011).

Despite the continued attention to BDNF, it is unknown if the trophic effects of BDNF signaling during the exercise regimen setup changes in the brain or if the rapid effects of BDNF-TrkB signaling during cognitive testing provide activational changes in the brain to support exercise-induced enhancements in learning and memory. Inhibition of BDNF-TrkB signaling during the exercise period can test for the importance of the trophic effects of BDNF on cognition. Using immunoadhesion chimera against the TrkB receptor (TrkB-IgG), mBDNF can be functionally sequestered to reduce signaling

through TrkB receptors. Unilateral intrahippocampal treatments of TrkB-IgG just prior to three days of voluntary exercise attenuated exercise-induced enhancements in spatial learning and memory and reversed the exercise-induced increases in BDNF and TrkB receptor mRNA (Vaynman et al., 2004). Thus, signaling through the TrkB receptor during exercise seems to prime the brain for enhanced future learning. However, it remains unclear whether the lack of mBDNF signaling imposed by the TrkB IgG during the exercise regimen decreased BDNF expression prior to learning, which, in turn, would lead to decreased BDNF-TrkB signaling during cognitive testing. Bekinschtein et al., 2011, proposed that the increased BDNF signaling during cognitive testing, in itself, produces improved learning and memory in physically fit animals (see commentary by Bekinschtein et al, 2011). A major goal of the current study was to test if BDNF-TrkB signaling during learning is supporting exercise-induced learning enhancements.

Here, we assessed the effects of voluntary wheel running on hippocampus- and striatum-sensitive learning, mBDNF content, and signaling. The first experiment evaluated the effects of three weeks of voluntary wheel running on learning place and response versions of a maze and on mBDNF content in the hippocampus, striatum, frontal cortex, cerebellum, and olfactory bulb of untrained rats. The second experiment used intrahippocampal and intrastriatal infusions of a TrkB inhibitor, K252a, just prior to maze training to test if BDNF-TrkB signaling during learning contributes to exercise-induced enhancements of cognition.

METHODS

Subjects

Three-month-old, male Sprague-Dawley rats were obtained from Harlan Laboratories. Upon arrival, rats were housed singly in clear plastic cages (~48 cm L x 26 cm W x 21 cm H) with free access to food and water and kept on 12/12 hr light/dark cycle.

Experiment 1 timeline. Rats were randomly assigned to sedentary (Sed) or voluntary exercise (Ex) experimental groups. At least one week after arrival in the vivarium, rats in the Ex group were placed in a cage with free access to a running wheel (Figure 1) for three weeks. Sed rats remained in their home cage. During the last seven days of the exercise period, rats were placed on food restriction to reduce body weights to 80-85% of free-feeding weights. Place task or response task training (described below) took place 21 days following running wheel access. Thus, there were two activity conditions for each task: Ex (n=10 for place learning; n=14 for response learning) and Sed (n=11 for place learning; n= 12 for response learning). Immediately after training, rats were overdosed and brain tissue was harvested for analysis of mBDNF concentration. An untrained subset of rats (sedentary n=9; exercise n=8) was killed 21 days following wheel access without training on the place or response tasks to determine differences in the levels of brain mBDNF between exercise and sedentary conditions in rats that had not received maze training.

Experiment 2 timeline. At least two days after arrival and acclimation to the vivarium, rats underwent cannula surgery. One week later, Ex rats were placed in clear plastic cages with free access to running wheels for three weeks. Sed rats remained in

their original cages. During the last 7 days of the exercise period, rats were placed on food restriction to reduce body weights to 80-85% of free feeding levels. Three weeks after access to running wheels, rats were trained on the place or response task. There were four experimental groups for each training task. Rats trained on the place task received intrahippocampal infusions of the TrkB antagonist K252a or vehicle 30 min prior to training; the following treatment groups were tested for place learning: Ex 1) vehicle (n=13) or 2) K252a (n=11), and Sed 3) vehicle (n=10) or 4) K252a (n=8). Rats trained on the response task received intrastriatal infusions of K252a or vehicle 30 min prior to training; the following treatment groups were tested for response learning: Ex 1) vehicle (n=7) or 2) K252a (n=7), and Sed 3) vehicle (n=7) or 4) K252a (n=8). Immediately after training, rats were overdosed and brain samples were harvested to assess cannula placement.

Running wheels

All rats in the Ex groups were singly housed and given access to running wheels (Lafayette Instruments Co.) for 21 days. Running wheels were constructed of stainless steel with a 35.5 cm diameter, with a mesh patterned running surface with a width of 10.9 cm. The running surface was composed of 1.5 mm rods connected by 7.9 mm centers with a 6.3 mm gap. Transparent plastic walls enclosed the wheels. A rectangular guillotine-style door was built into the wheel and connected to the cage to restrict or allow access into and out of the wheel. The wheel sat above a steel tray (~40.5 cm L x 36 cm W) to collect animal debris. The cages were attached to wheels via short stainless steel tunnels (Figure 2.1). In a separate room, a computer recorded running distances. Wheel revolutions were tracked using the Activity Wheel Counter

(Lafayette Instruments Co.). Each wheel revolution was equal to 1.1 meters. Data were collected once every four hours and analyzed off-line.

Cannula implantation

Rats in Experiment 2 were anesthetized with 2-3.5% isoflurane and received 30,000 units of penicillin (i.m.; Dura-Pen; Henry Schein, Inc., Indianapolis, IN, USA) and injections of Rimadyl (5 mg/kg, s.c.) for analgesia prior to stereotaxic surgery. Sterile, stainless steel guide cannulae (22 gauge, 6 mm; Plastics One, Inc., Roanoke, VA, USA) aimed either at the dorsal hippocampus (AP -3.8, ML \pm 2.5, -1.9 mm ventral to dura) or the dorsal striatum (AP +0.2, \pm ML 3.6, -2.8 mm ventral to dura), were implanted bilaterally in all rats in Experiment 2 (Figure 2.2). Coordinates were chosen based on reports from our lab demonstrating drug and hormone actions in the hippocampus and striatum on place and response learning (Zurkovsky et al., 2006; 2007; 2011; Newman et al., 2011) and were adapted from the atlas of Paxinos and Watson (1986). Four stainless steel screws were placed into the skulls for anchors and the assemblage was cemented in place with dental acrylic. To keep cannulae open, 28 gauge stylets (Plastics One, Inc.) cut to the length of the guide cannulae were inserted at the time of surgery, checked daily, and removed only during the central infusions.

Drug infusion

In Experiment 2, rats trained on the place maze received intrahippocampal K252a or vehicle infusions while those trained on the response maze received intrastriatal infusions. Thirty min prior to training, rats received bilateral infusions (0.5 μ l in 1 min) of vehicle (50% dimethyl sulfoxide (DMSO) and artificial cerebrospinal fluid (aCSF) in mM: 128 NaCl, 2.5 KCl, 1.3 CaCl₂, 2.1 MgCl₂, 0.9 NaH₂PO₄, 2.0 Na₂HPO₄, 1.0 dextrose) or

K252a (25 μ M) in 50% DMSO/aCSF. A CMA microdialysis infusion pump was used to deliver K252a or vehicle through a 29-gauge needle attached to a 10 μ l Hamilton syringe. The needle was left in place for 1 min to await diffusion away from the infusion site.

Place and response maze training

Rats were trained on either a place or response task. To motivate maze learning, all rats were food-restricted to 80-85% of their free-feeding weight during the seven days prior to training. In addition to receiving their daily ration of rat chow, rats also received Frosted Cheerios ©, the food to be used later as the reinforcement during maze training, to reduce neophobia during training.

Place and response training were done using a four-arm, plus-shaped maze configured into a “T”, similar to tasks used previously (Korol and Kolo, 2002; Canal et al., 2005; Zurkovsky et al., 2007; 2011). Both tasks require rats to determine the location of a food reward (half of a Frosted Cheerio ©). Rats trained on the place task learned to locate the food reward based on extramaze cues (i.e. east or west arms; Figure 2.3A) while rats trained on the response task learned to locate the food reward using egocentric body movements (i.e. right or left turns; Figure 2.3B).

Rats were trained between three and eight hours after the start of the light cycle. Untrained rats used in Experiment 1 were treated the same as place- and response-trained rats but were not exposed to the maze environment or training. Thus, these untrained rats were exposed to sedentary or exercise conditions for 21 days, and food-restricted for seven days prior to sacrifice, which occurred between three and eight hours after the start of the light cycle.

Training apparatus and environment

The full length of each alleyway composing the T-maze was 104 cm. Each of the four arms was 13 cm wide and 18 cm high. Each individual arm was 46 cm in length and the center of the maze (where all four arms converged) was 12 cm². The maze walls were constructed of black, ¼ in Plexiglas® and the floor of black, matte Plexiglas®. Mazes were affixed to a rotating platform (46 cm diameter) that enabled the rotation of the maze between trials using preset stops at 90° intervals. In addition, plastic food receptacles with inaccessible food rewards were attached to the ends of each maze arm. Odor cues of the food reward in each arm and rotation of the maze between trials diminished the use of inadvertent intramaze cues to solve the task. In the place training condition, the room contained several 2- and 3-dimensional extramaze cues. In the response training condition, ceiling-to-floor curtains surrounded the maze to minimize extramaze visual cues. A lamp with indirect lighting was placed in each corner of the room provided symmetrical ambient light. A fan was turned on to mask building noise.

Training procedure

On the day of training, rats were placed in a cage with fresh bedding and without food or water and were left in the training room for 15 min to allow acclimation to the training environment. For each rat, start arms were quasi-randomized across twenty trials, such that each start arm (“north” or “south”) was used for 10 trials. For the place task, the goal location (east or west) and for the response task, the direction of body turn (right or left) was counterbalanced across rats within a treatment condition.

A trial started when a rat was placed in the start arm and ended after all four paws entered the choice arm. When the rat chose the reward arm, it remained there until eating was complete and was removed from the maze before exiting the choice arm. If the rat chose the incorrect arm, it was allowed to remain for approximately 5 sec or until it turned to exit, before being returned to the holding cage for the intertrial interval of 30 sec. Maximum choice time was 2 min; if a rat failed to make a choice it was returned to the cage for the inter-trial interval and marked as an omission. Omissions were not included in the total number of recorded correct and incorrect choices.

All rats in Experiment 1 were trained to 75 trials. In Experiment 2, place trained rats were trained to 100 trials and response trained rats were trained to 75 trials. Immediately after training, rats received an overdose of sodium pentobarbital (75 mg/kg). After reflexive movements were no longer evident, rats were decapitated (Experiment 1) and tissue was collected for biochemical assessment. Rats in Experiment 2 were transcardially perfused and brains were collected for determination of cannula placement.

Post-sacrifice tissue preparation

Experiment 1. Immediately after training, brains were harvested, dissected on ice, flash-frozen within 150 sec with dry ice, and stored at -80°C. The samples were homogenized on ice in a lysis buffer (58 g NaCl, 157.6 g Tris-HCl, 65.01 mg NaN₃, dissolved in 1 L deionized water) and dissolved protease inhibitor tablets (Roche Diagnostic Corporation, Indianapolis, IN). Specifically, brain samples from hippocampus, striatum, frontal cortex, cerebellum and olfactory bulb with lysis buffer

were added to borosilicate glass tubes (Wheaton Industries, Inc., Millville, NJ) and pulverized 25 times using polytetrafluoroethylene pestles (Thomas Scientific, Swedesboro, NJ). After homogenization, the samples sat on ice for 5 min, were transferred to a microfuge tube, and were centrifuged with a Centra MP4R (International Equipment Company, Nashville, TN) at 4°C for 15 min at 6,700 x g. The supernatant was collected and stored at -20°C until assayed for protein content and mBDNF using an enzyme-linked immunosorbent assay (ELISA).

Experiment 2. Following transcatheter perfusion with 0.1 M phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA), brains were removed and stored in 4% PFA in 0.1 M phosphate buffer overnight and then cryoprotected with 20% glycerol. Brains were sectioned for cannula histology. Sections containing cannula tracts were mounted on gelatin-coated slides and stained with cresyl violet. Sections were inspected for cannula placement and damage extending beyond the cannula site. Only rats with accurate bilateral cannula placements and without excessive damage were included in the final analyses (n=5) (Figure 2.2).

Protein assay

The protein concentrations of the samples were determined using a Micro-BCA assay kit (Pierce, Rockford, IL). Each tissue sample was diluted 1:3 with the lysis buffer. Standards (25 µl) ranging from 0 µg/ml to 2000 µg/ml and diluted samples (25 µl) were added in triplicate to a 96-well microtiter plate. A working reagent (100 µl) was added to each well and incubated at 37°C for 30 min. After incubation, the plate was cooled to room temperature (21°C). Absorbance at 570 nm was then measured in a ThermoMax microplate reader using Ascent software (Fischer Scientific, Pittsburgh,

PA).

BDNF ELISA

Sandwich-style ELISAs were performed using the BDNF Emax ImmunoAssay System kit (Promega, Madison, WI) according to manufacturer's instructions to detect mBDNF. The absorbance was read at a wavelength of 450 nm (within 30 min of stopping the reaction) in the ThermoMax microplate reader with Ascent software (Fischer Scientific, Pittsburgh, PA).

mBDNF content was interpolated from standard curves conducted on each plate (ranging from 0 –500 pg/ml for BDNF). To adjust for differences in the total levels of protein across samples, the mBDNF protein value was divided by the total sample protein value to determine pg of mBDNF per μ g of protein. To compare values across experiments and brain regions, concentrations were converted to percent change of sedentary controls.

To determine the recovery rates of mBDNF for the homogenization protocol across the brain regions assayed, a spike and recovery assay was performed on the hippocampus, striatum, frontal cortex and cerebellum samples. Spiked samples were compared to unspiked samples and the recovery rates were calculated. Our spike and recovery assay revealed a 69% recovery rate for the hippocampus, a 62% recovery rate for the striatum, a 55% recovery rate for the frontal cortex, a 73% recovery rate for the cerebellum.

Statistical analysis

A repeated measure one-way ANOVA was used to compare running distances across the 21-day period in striatum- and hippocampus-implanted rats.

Learning was defined by the number of trials to reach the criterion of 9 correct trials out of 10 with at least 6 consecutive correct trials, and by percent correct across blocks of training trials. Because some rats in Experiment 1 failed to reach the learning criterion within the maximum of 75 trials given, the data were not normally distributed. Therefore, non-parametric Mann-Whitney tests were performed to make pair-wise comparisons of the exercise treatment. A repeated measure one-way ANOVA was used for accuracy across place and response learning. Simple regressions were performed to test for correlations between running distance and trials to criterion in the place and response tasks. The effects of the exercise treatment on the mBDNF content in the hippocampus, striatum, frontal cortex, cerebellum and olfactory bulb were analyzed using Student's *t*-tests.

In Experiment 2, the main effects of the K252a and exercise treatments on trials to reach criterion in place and response learning were analyzed using non-parametric Mann-Whitney tests to make comparisons across drug and exercise treatments.

Statistical significance was set at $\alpha = 0.05$.

All procedures followed the institutional and federal guidelines and were approved by the IACUC at the University of Illinois Urbana Champaign.

RESULTS

All rats given access to a running wheel ran substantial distances in their wheels. On average, the distance run per week increased dramatically throughout the exercise

period. In particular, during the third week of wheel running when rats were placed on a 7-day period of food restriction, the rats significantly increased their running distances. On average, rats in Experiment 1 ran about 5 km the first week, 7 km the second week, and 20 km during the third week during food restriction (Figure 2.4A). Hippocampus-implanted rats (Figure 2.4B) in Experiment 2 ran distances similar to those of unimplanted rats in Experiment 1. However, striatum-implanted rats ran significantly ($F_{(1,20)}=2.63$, $p<0.01$) shorter distances overall compared to other rats (Figure 2.4C). All rats showed qualitatively similar changes in running across the three-week exercise period (Figure 2.4A, 2.4B, 2.4C).

Three weeks of voluntary access to running wheels enhanced the speed of acquisition in both the place and response versions of the mazes. Acquisition of the place task was faster in the rats allowed to exercise for 21 days. The Ex rats demonstrated significantly fewer trials to reach criterion than did the Sed rats ($U_{10,11}=18.5$; $p<0.05$; Figure 2.5A). The exercise-induced enhancement in learning was also seen in the striatum-sensitive response task. There was a significant decrease in the number of trials to reach criterion in the Ex compared to the Sed rats ($U_{12,14}=38$, $p<0.05$; Figure 2.5C).

The learning curves reflecting changes in percent correct across training revealed exercise-mediated increases in speed of learning that aligned with reductions in trials to criterion. We found a main effect of treatment in rats that were place trained (Ex vs. Sed; $F_{(1, 19)}=8.06$; $p<0.01$; Figure 2.5B) such that the Ex group had more correct trials across the training session than did the Sed group. Parallel findings were reported in the response task, with a slight difference between the exercise treatments and

accuracy across training ($F_{(1,23)}=1.92$; $p<0.2$; Figure 2.5D). For both place and response learning tasks there was a reliable increase in the number of correct trials across the individual training sessions for all rats regardless of exercise condition supported by a main effect of trial block (Figure 2.5B, $F_{(14, 266)}=10.22$; $p<0.001$; Figure 2.5D, $F_{[6,138]}= 41.5$; $p<0.0001$).

Examination of the learning curves for rats completing place (Figure 2.5B) and response (Figure 2.5D) tasks reveals two different patterns of effects of exercise. For place learning, differences between Ex and Sed rats emerged late in training, seen by significant differences between exercise conditions in percent correct in the second half of training (first half: $F_{(1,20)}=0.12$, $p>0.2$; second half: $F_{(1,20)}=8.8$, $p<0.01$). In contrast, exercise-related differences in the response task were evident at the onset of training, with significant differences between groups observed only in the first half of training (first half: $F_{(1,23)}=4.03$, $p\leq 0.05$; second half: $F_{(1,23)}=1.8$, $p<0.2$).

The running distances of place trained rats correlated significantly with trials to criterion in the place task ($R^2=-0.52$, $p<0.02$; Figure 2.6A). Rats that ran farther distances over the 3-week running period learned the place task in fewer trials than rats that ran less. The same was true for the response task, running distances correlated significantly with trials to criterion on the response task ($R^2=-0.44$, $p<0.01$; Figure 2.6B). Rats that ran the farthest during the 3-week running period learned the response task in fewer trials than rats that ran less.

In general, brains from Ex rats had increased tissue content of mBDNF per unit protein compared to tissue from Sed rats. Significant elevations in concentrations of mBDNF were observed in the striatum and cerebral cortex, but not the hippocampus,

cerebellum, or olfactory bulb, each of which showed increases that did not reach statistical significance (Figure 2.7). Specifically, the Ex rats had approximately 25% more mBDNF in the hippocampus compared to the Sed controls but this increase did not reach significance ($t_{13}=1.63$, $p\leq 0.1$; Figure 2.7A). Compared to Sed rats, Ex rats had more than 50% increased concentrations of mBDNF in the striatum ($t_{14}=2.75$, $p\leq 0.01$; Figure 2.7B) and frontal cortex ($t_{12}=3.7$, $p\leq 0.01$; Figure 2.7C). In the cerebellum, Ex rats had up to 40% more mBDNF compared to the Sed controls but this increase also did not reach statistical significance ($t_{14}=1.77$, $p\leq 0.1$; Figure 2.7D). The mBDNF content of the olfactory bulb in Ex rats was higher than, but not statistically different from levels found in Sed rats ($t_{10}=0.68$, $p>0.2$; Figure 2.7E). Raw values of mBDNF in the hippocampus averaged 44 ± 8 (pg BDNF/mg protein) in exercise and 32 ± 5 (pg BDNF/mg protein) in sedentary rats. In the striatum, mean values of mBDNF were 17.7 ± 1.7 (pg BDNF/mg protein) in Ex rats and 14.8 ± 1.6 (pg BDNF/mg protein) in the Sed rats.

Results from Experiment 1 demonstrate that three weeks of voluntary wheel running enhances learning in both the place and response versions of a 4-arm maze. In addition, voluntary exercise led to global increases in mBDNF compared to the Sed rats. We tested if/how increased mBDNF plays a role in the exercise-induced benefits in place and response learning by blocking BDNF-TrkB signaling during learning.

Blockade of TrkB signaling by K252a attenuated the robust enhancements in place and response learning by exercise, but did not impair learning in sedentary rats. For the place task, pair-wise Mann-Whitney analyses on trials to criterion data revealed significant effects between groups (Figure 2.8A). Replicating our findings from

Experiment 1, the Ex vehicle-treated rats showed a significant reduction in the number of trials to reach criterion compared to the Sed vehicle-treated rats ($U_{10,13}=33$, $p\leq 0.05$). Ex rats treated with intrahippocampal K252a required significantly more trials to reach criterion compared to Ex rats treated with vehicle ($U_{13,11}=31$; $p<0.05$; Figure 2.8A). Furthermore, Ex K252a-treated rats learned the place task after a similar number of trials as did the Sed vehicle-treated control rats. Importantly, there was no significant difference in the trials to reach criterion between Sed rats treated with K252a and Sed rats treated with vehicle ($U_{10,8}=24$, $p>0.05$).

Parallel results were obtained following striatal treatments in the response task, with an even more robust enhancement of learning in rats with voluntary access to wheels. The Ex rats with intrastriatal vehicle treatments learned the response task in fewer trials than did the Sed rats with vehicle treatments ($U_{(7,7)}=2$; $p<0.01$; Figure 2.8B). As observed with intrahippocampal K252a and place learning, intrastriatal K252a treatment reversed the exercise-induced enhancement in response learning. Ex rats receiving K252a infusions required significantly more trials to learn the response task compared to Ex rats with vehicle treatments ($U_{(6,7)}=3$; $p\leq 0.01$; Figure 2.8B). Surprisingly, in Sed rats, intrastriatal K252a infusions produced a significant reduction in trials to criterion (Sed-vehicle vs Sed-K252a, $U_{(7,8)}=5.5$; $p<0.01$; Figure 2.8B).

Thus, for the place task, hippocampal K252a attenuated the exercise-induced enhancements in learning and had no effect on learning in the sedentary rats. In the response task, K252a treatment also attenuated exercise-induced enhancements in learning, but in addition, enhanced learning in sedentary rats.

DISCUSSION

Taken together with previous studies, these findings suggest that the exercise-induced enhancements in cognition are modulated by BDNF-TrkB signaling at the time of learning.

The place learning enhancements induced by physical activity support previous findings showing exercise-induced improvements in spatial learning or memory using the 8-arm radial maze (Anderson et al., 2000), the swim task (Fordyce and Farrar, 1991), spontaneous alternation (Hall et al., 2014), object recognition (Hopkins et al., 2010; O'Callaghan, et al., 2007), and the Barnes maze (Wong-Goodrich et al., 2010; Jacotte-Simancas et al., 2013). The exercise-induced enhancements in response learning fit into earlier work showing that physical activity increases angiogenesis and BDNF protein content in the striatum (Marais et al., 2009; Ding et al., 2004). Also in the current study, exercise robustly increased mBDNF concentrations in the striatum, likely supporting increases in BDNF-TrkB signaling during response learning in exercise rats.

Rats with striatum implants (in Experiment 2) ran less than unimplanted rats in Experiment 1 and hippocampus-implanted (Experiment 2) rats. All rats did show increased running distances from the first to the third week of wheel access. However, the fact that the enhancement of response learning was particularly robust in Experiment 2 despite the lower running distances also suggests that even relatively low levels of exercise support response learning enhancements. In Experiment 1, running distances over the 3-week running period correlated with place and response learning scores. In addition, others have found that increased running does correlate with increases in BDNF mRNA expression (Neeper et al., 1996) and protein levels in the

hippocampus (Johnson et al., 2003; Cotman and Berchtold, 2002). Thus, there may be a threshold level of physical activity necessary to facilitate striatum-sensitive learning.

In untrained physically active rats, mBDNF concentrations increased in all regions measured but the increases only reached statistical significance in the striatum and frontal cortex. The mechanism for BDNF degradation is guided by sortilin (Evans et al., 2011) and higher quantities of sortilin are found in the hippocampus and olfactory bulb than in the striatum, frontal cortex, or cerebellum (Sarret et al., 2003). Thus, it is possible that mBDNF degrades more rapidly in the hippocampus and olfactory bulb than in other brain regions, leading to less robust exercise-induced differences in tissue content.

Robust increases in BDNF protein and BDNF mRNA in the hippocampus have been reported after one week or more of wheel running (Neeper et al., 1996; Oliff et al., 1998; Russo-Neustadt et al., 2001; Adlard et al., 2005). It is possible that longer or more intense exercise exposures are necessary to see significant elevations in the hippocampus; rats used here ran relatively little in the first week with running distances increasing incrementally over the three-week access period. Furthermore, previous studies tested for total BDNF protein and mRNA while our assays measured the cleaved, mature form of BDNF. Of note, a large amount of BDNF found in the hippocampus is made and stored in the premature state as proBDNF until sufficient activation cleaves proBDNF into mBDNF for release (Nagappan, et al., 2008). Thus, it is possible that exercise-induced increases in hippocampal BDNF synthesis and storage reported by others (Neeper et al., 1996; Adlard et al., 2005) supports increased BDNF release during learning. If we had taken measurements of mBDNF protein during

or following place learning it is likely that we would have seen increases in hippocampus mBDNF levels in exercise rats compared to sedentary rats. It is unclear if increased mBDNF in the striatum reflects an increase in mBDNF storage, allowing for increased BDNF release during response learning or if mBDNF is constantly elevated and released in the striatum of physically fit rats, regardless of learning. Currently studies are underway to measure BDNF release in the hippocampus and striatum of exercise and sedentary rats before, during, and following place and response learning. Given the findings of the current and past studies, we hypothesize that during place learning BDNF release is increased in the hippocampus of exercise rats.

More direct evidence for a role of BDNF in exercise-induced facilitation of learning comes from our findings from Experiment 2. K252a treatment attenuated learning enhancements in physically active rats and did not impair learning in sedentary rats. Our findings with K252a suggest that exercise exerts its cognition-enhancing effects through BDNF-TrkB signaling at the time of training; regardless of the status of previously induced neuroplasticity such as neurogenesis and angiogenesis (van Praag et al., 1999; Isaacs et al., 1992; Marais et al., 2009; Ding et al., 2006). Our working model is that three weeks of wheel running increases mBDNF content, which in turn, supports increases in BDNF-TrkB signaling during learning, thus enhancing learning. The importance of BDNF signaling for supporting learning and memory processes have been repeatedly shown in previous studies. For example, treatment with anti-sense BDNF oligonucleotides impairs reference and working memory in the radial arm maze (Mizuno et al., 2000) and memory in the inhibitory avoidance task (Ma et al., 1998). In addition, pre training infusions of anti-BDNF antibodies impairs memory formation in the

passive avoidance (Alonso et al., 2002) and inhibitory avoidance task (Alonso et al., 2005). Taken together with past studies, the current findings highlight the necessity of BDNF-TrkB signaling during, not prior to, learning to support exercise-induced learning enhancements.

BDNF-TrkB signaling has long been regarded as a potent modulator of learning and memory. There are many reports of increased BDNF mRNA expression, mBDNF protein, and TrkB phosphorylation during and following learning in the brain areas specific to acquisition of particular tasks (Ma et al., 2011; Kim et al., 2012; Gomez-Pinilla et al., 2007; Alonso et al., 2002; Kesslak et al., 1998). When mBDNF binds to TrkB pre-synaptically, it modifies neurotransmitter release and potentiates synaptic transmission (Gottschalk et al., 1998). Postsynaptically, mBDNF increases intracellular Ca^{2+} concentrations and induces gene expression of proteins such as synaptobrevin, synaptophysin (McAllister et al., 1999), synapsin (Vaynmann et al., 2004), Ca^{2+} /Calmodulin-dependent protein kinase II (CamKII) (Vaynman et al., 2007) and importantly, the TrkB receptor (Liu et al., 2009; Vaynman et al., 2004). Because exercise increases BDNF -TrkB signaling, physically fit rats have upregulation of the TrkB receptor, BDNF, and intracellular components of the TrkB signaling cascade (Vaynmann et al., 2004; Vaynmann et al., 2006; Molteni et al., 2002; Vaynmann et al., 2007). Thus, the increase in baseline mBDNF levels in physically fit rats may allow for enhanced BDNF-TrkB signaling during learning in the brain areas selectively activated by physical activity.

Despite strong evidence that BDNF signaling is indeed involved in memory formation (for review see Tyler et al., 2002), we found no learning impairments following

K252a treatment in sedentary rats. One possibility is that prior studies finding K252a-induced learning deficits assessed the durability of memory formation and not learning *per se* (Ou et al., 2010; Liu et al., 2008; Kim et al., 2012). As such, K252a treatment into the hippocampus and striatum in sedentary rats would decrease later memory for place and response learning, respectively, if tested after relatively long intervals. Surprisingly, K252a did not simply fail to impair place and response learning in sedentary conditions, but in fact improved response learning compared to vehicle-treated rats. The K252a-induced response learning enhancement may be due to the non-specific effects of K252a. K252a can interfere with TrkA and other protein kinases, particularly at high doses (Tapley et al., 1992; Towmey 1990; Kase et al., 1986). However, only a single dose of K252a was used to block TrkB signaling in the current experiment; it is possible that a higher or lower dose would impair place or response learning in sedentary rats. A more extensive study of K252a effects on cell signaling is necessary to elucidate its non-specific actions in the hippocampus and striatum of physically active and sedentary rats.

Specific blockade of TrkB during learning, but not during the exercise regimen, is necessary to substantiate the role of BDNF-TrkB signaling in exercise-induced learning enhancements. Unfortunately, the specific inhibitors of TrkB (TrkB-IgG) need at least a few days for dispersal throughout the brain region of interest (Altun et al., 2001). In the current study, TrkB blockade with K252a treatment was restricted to the learning period. Since, it is not known how much TrkB inhibition occurs during the TrkB-IgG dispersal period (2-3 days) prior to learning, the use of TrkB-IgG could produce a prolonged pre-

learning disruption of TrkB signaling during the last 2-3 days of exercise, which may alter BDNF-TrkB signaling during learning, thus confounding our experimental design.

Hippocampal neurogenesis receives considerable attention as the anatomical mechanism for improved cognition in physically fit rats (van Praag et al. 1999; 2005; 2008; Perieira et al., 2007; Cotman et al. 2007). However, our findings indicate that neurogenesis alone is not necessary to support exercise-mediated cognitive enhancements, a prospect proposed previously (Bekinschtein et al., 2011). We showed robust exercise-induced enhancements in striatum-sensitive response learning but the striatum has little, if any, demonstrable adult neurogenesis. Moreover, acute treatment of K252a just prior to training blocked the exercise-induced place learning enhancements despite the increase in hippocampal neurogenesis that wheel running presumably stimulated (van Praag et al., 1999; 2005). Given that BDNF increases hippocampal neurogenesis (Scharfman et al., 2005); a more parsimonious explanation is that exercise-induced neurogenesis is a byproduct of exercise-induced increases in BDNF-TrkB signaling. Taken together, the data from the current and past studies suggest that exercise enhances hippocampal- and striatal-sensitive learning, not by increases in hippocampal neurogenesis or angiogenesis, but instead via the signaling events associated with BDNF-TrkB during learning, such as increased neurotransmitter release (Knipper et al., 1994; Sala et al., 1998; Santafe et al., 2014; Tyler and Pozzo-Miller, 2001; Numakawa et al., 1998; Lessman et al., 1994; Du and Poo, 2004) and/or potentiated BDNF TrkB signaling (Vaynman et al., 2003; Cassilhas et al., 2012).

FIGURES

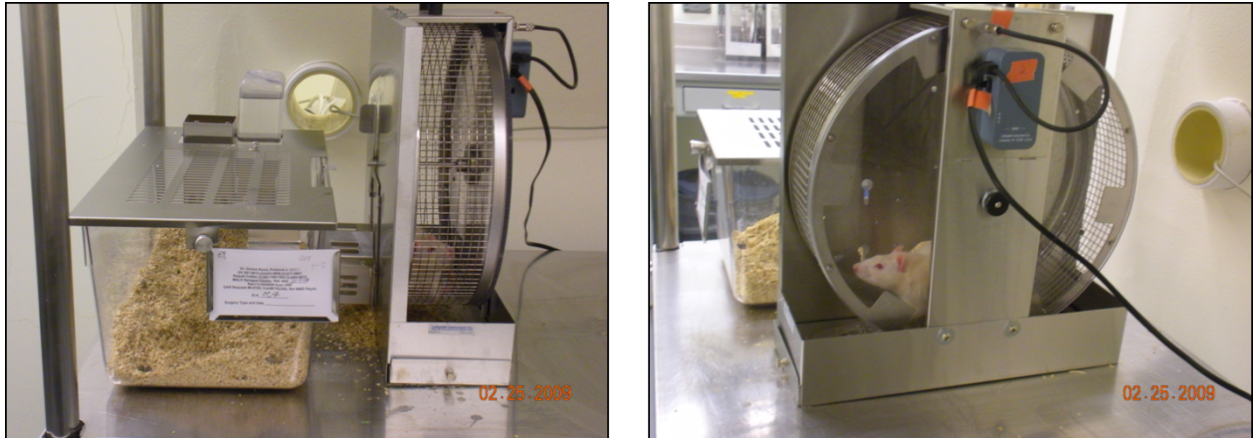


Figure 2.1. Running wheel apparatus. Rats had free access to the running wheel through a tunnel from their home cages.

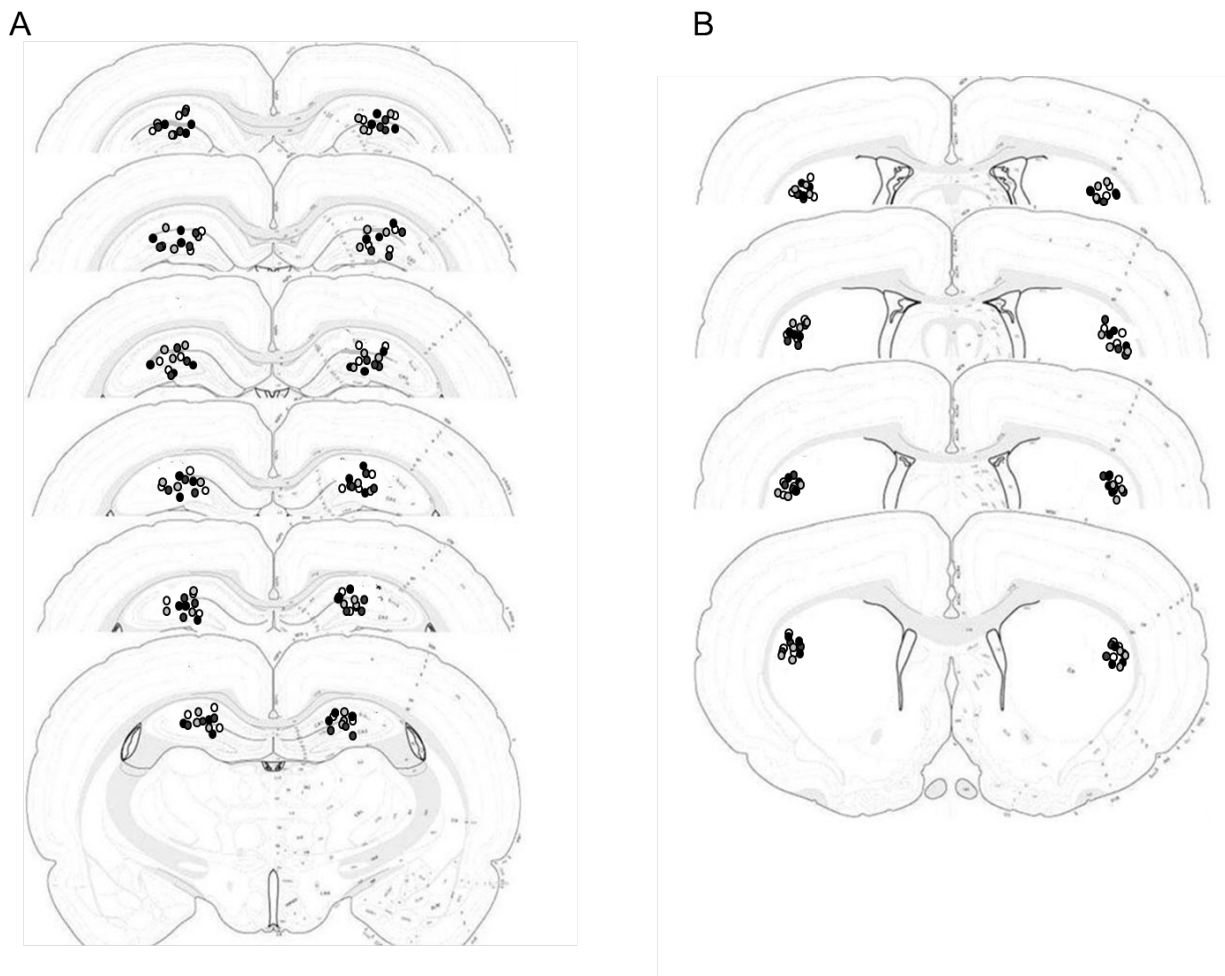
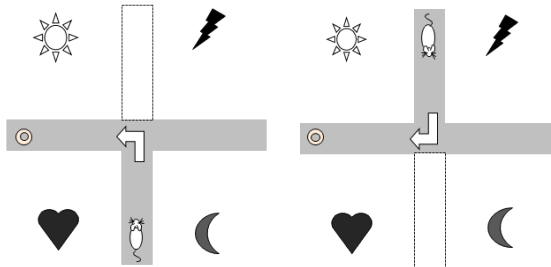


Figure 2.2. Cannula placements in the dorsal hippocampus and the dorsal lateral striatum. Rats in all treatment groups had similar implantation sites in A) the dorsal hippocampus and B) the dorsal lateral striatum. Shaded circles represent cannula placements for each experimental group. White= Sed + Vehicle; light gray= Sed + K252a; dark gray= Ex + Vehicle; black= Ex + K252a.

A

Place Learning



B

Response Learning

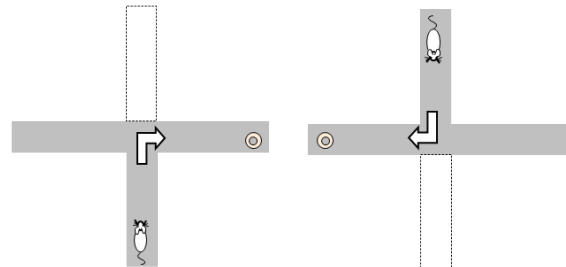


Figure 2.3. Place and response mazes. Maze environments used for A) place and B) response training. Place training (A) occurred on a maze in a room with extramaze cues available for the rats to find their food rewards. In the place task the rats were trained to find the rewards in the same locations in the room. Response training (B) occurred in a room with no extramaze cues as the rats were trained to find their food rewards through their use of egocentric body movements (i.e. left or right turns), regardless of the previous locations of the reward.

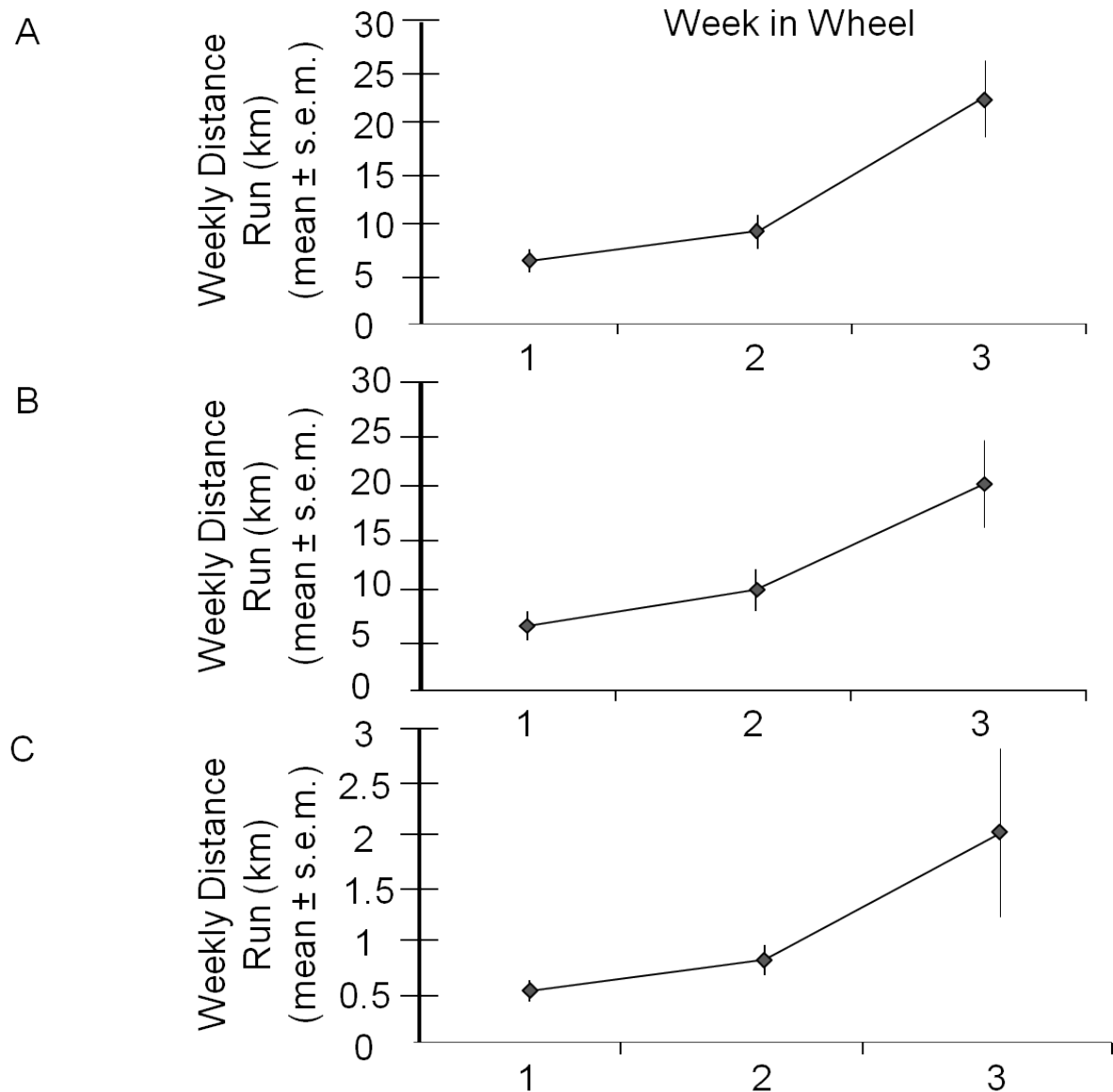


Figure 2.4. Total distance run over three weeks. Rats increased running distances with subsequent days of wheel access. All rats showed substantial increases in their running during the third week, representing the week of food restriction. A) Unimplanted rats from Experiment 1 and B) rats with bilateral cannula implants in the hippocampus from Experiment 2 ran similar distances over the three-week period. C) Rats with bilateral cannula implants in the striatum from Experiment 2 ran much less overall across the three-week period.

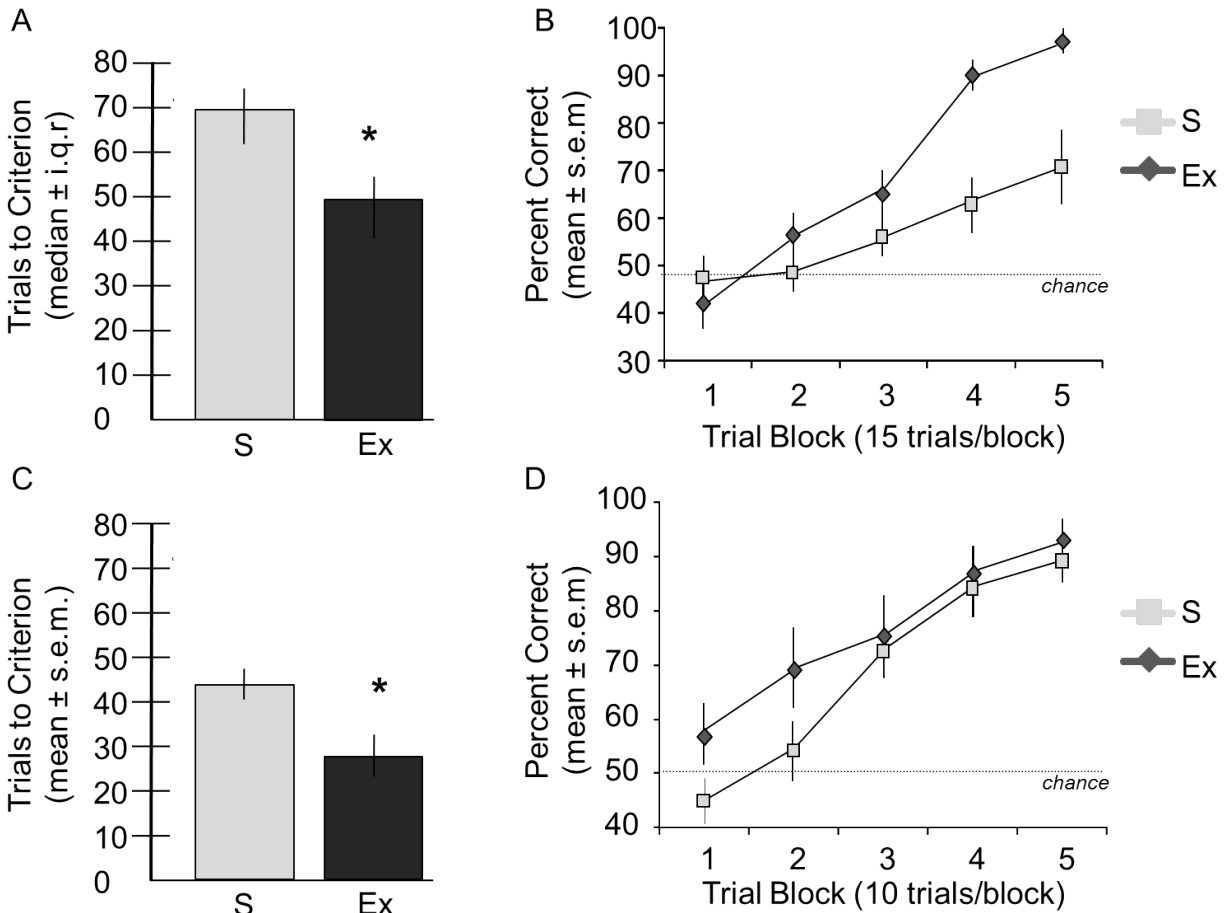


Figure 2.5. Place and response learning in physically active and sedentary rats.

The effect of 3 weeks voluntary exercise on A, B) place and C,D) response learning.

Exercise decreased the number of trials to learning criterion in both A) place and C) response tasks. Changes in accuracy over the training period for B) place training with trials grouped into 15-trial blocks, and for D) response training with trials grouped into 10-trial blocks. For place training Ex rats had steeper learning curves than did S rats, demonstrating faster learning. *= $p < 0.05$ vs Sedentary; Ex=exercise, S=Sedentary. In place-trained rats N=11 for S and N=10 for Ex. In response-trained rats N=12 for S and N=14 for Ex.

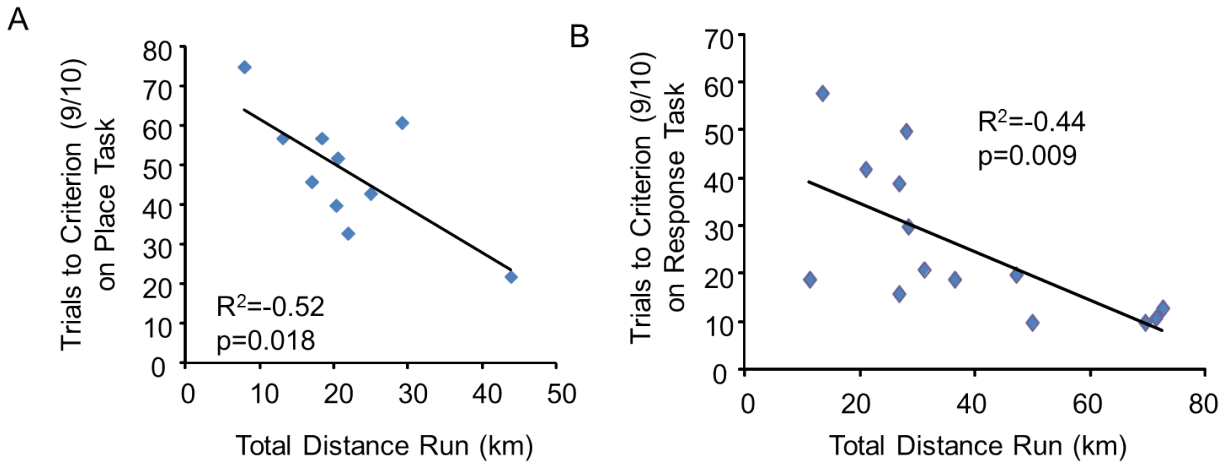


Figure 2.6. Correlations between running distance and trials to reach criterion in the place and response tasks. In A) the place task, rats that ran the farthest during the 3-week running period learned the place task in fewer trials than rats that ran less. In B) the response task, rats that ran the farthest during the running period learned the response task in fewer trials than rats that ran less.

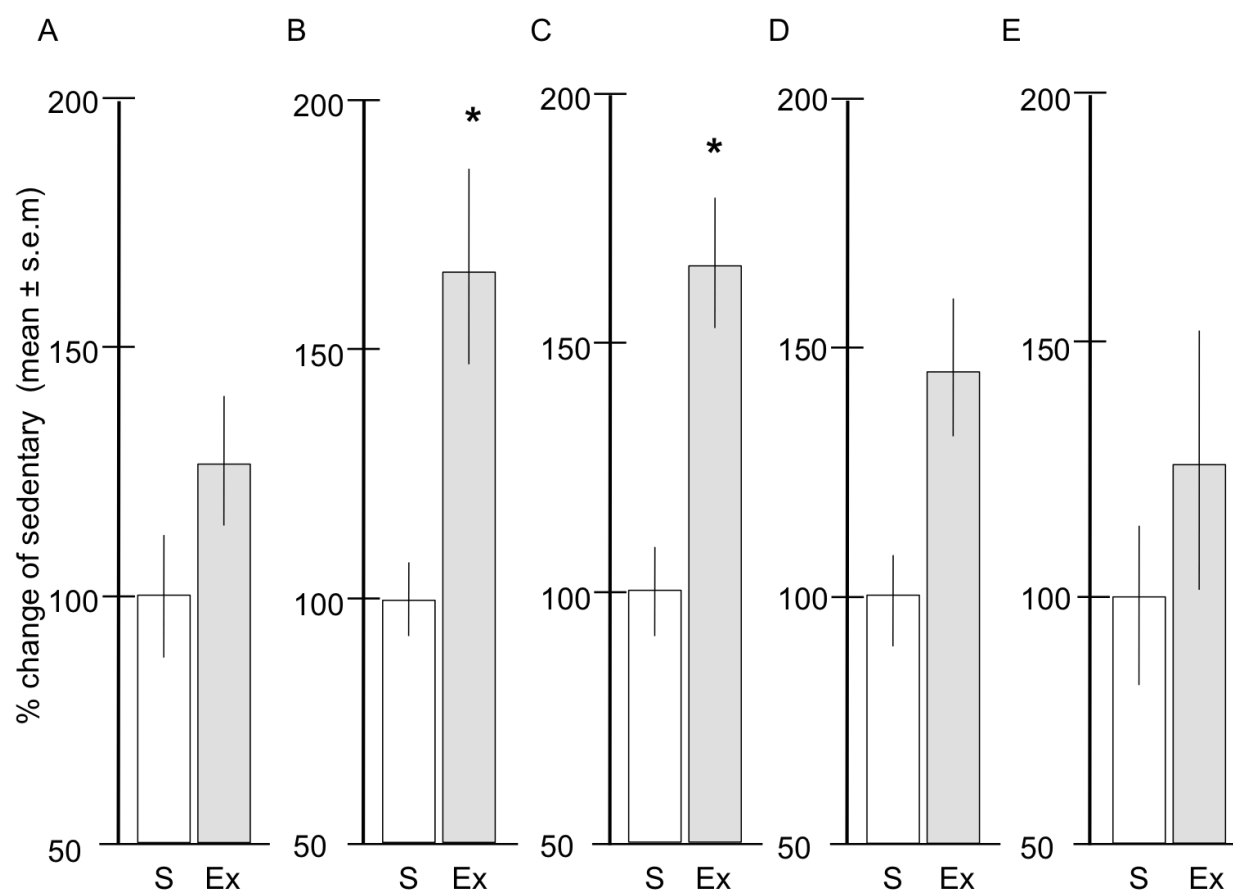


Figure 2.7. mBDNF content in hippocampus, striatum, frontal cortex, cerebellum and olfactory bulb in behaviorally naïve sedentary and physically active rats. A) Exercise tended to increase mBDNF levels in the hippocampus and D) cerebellum. In the B) striatum and C) frontal cortex, exercise significantly increased mBDNF compared to the sedentary controls. E) There was no significant effect of exercise in the olfactory bulb (E). * = $p < 0.05$ vs. Sedentary; Ex=exercise, S=Sedentary. N=9 for S, N=8 for Ex.

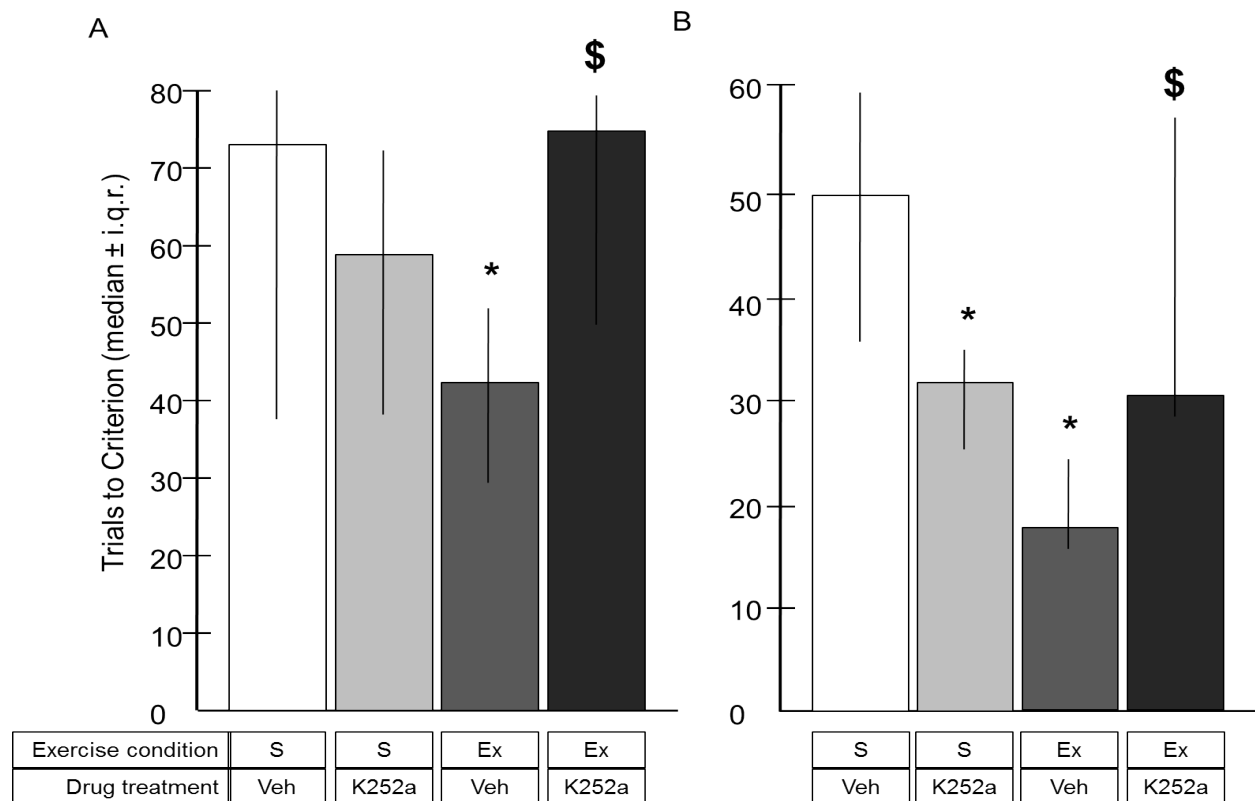


Figure 2.8. K252a treatment attenuated exercise-induced place and response learning enhancements. A) K252a treatment into the hippocampus prior to place training attenuated exercise-induced enhancements in place learning. Vehicle-treated Ex rats learned the place task in fewer trials than vehicle-treated Sed rats. B) K252a treatment into the striatum prior to response training attenuated exercise-induced enhancements in response learning. Vehicle-treated Ex rats learned the response task in fewer trials than vehicle-treated sedentary rats. Sedentary K252a-treated rats learned the response task in fewer trials than vehicle-treated sedentary controls. *=p<0.05 vs. sedentary vehicle-treated controls; \$=p<0.05 vs. exercise vehicle-treated. For place-trained rats N=10 for S-Veh; N=8 for S-K252a; N=13 for Ex-Veh; N=11 for Ex-K252a; for response-trained rats N=7 for S-Veh; N=8 for S-K252a; N=7 for Ex-Veh; N=7 for Ex-K252a.

TRANSITIONAL THOUGHTS

Our findings from Chapter 2 show that physical activity enhances place and response learning through BDNF-TrkB signaling. These findings support our theory of “Use it and Boost it”. Physical activity engages the hippocampus and striatum, thus leading to an increased reliance on BDNF-TrkB signaling, to support enhanced learning in the future. Exercise increases BDNF protein content in the hippocampus and striatum (Adlard et al., 2005; Berchtold et al., 2005; Chapter 2; Ding et al., 2004b). Cognitive activity, also increases BDNF protein expression in the areas engaged by the cognitive activity (Kesslak et al., 1998; Figure 1.2; Kim et al., 2012). Cognitive training also enhances learning and memory across a variety of tasks (Anguera et al., 2013; Ball et al., 2002; Light et al., 2010).

Given that both cognitive and physical activities enhance cognition and increase BDNF in the brain it is possible these activities are enhancing cognition via similar neural mechanisms. In the following chapter we tested if cognitive activity, like physical activity, enhances place and response learning, and if BDNF-TrkB signaling is underlying the cognitive activity-induced learning enhancements. The following set of studies is supporting our “Use it and Boost it” theory. Cognitive tasks directly engage neural systems so that in the future those neural systems will function better, leading to enhanced cognition.

CHAPTER 3:
USE IT AND BOOST IT: ENGAGEMENT IN A SPATIAL WORKING MEMORY TASK
ENHANCES SUBSEQUENT PLACE AND RESPONSE LEARNING THROUGH
BDNF-TRKB SIGNALING

ABSTRACT

Voluntary physical activity enhances place and response learning. In addition, these learning enhancements appear to be mediated by BDNF acting through its TrkB receptor. The present experiment determined whether similar effects were evident after cognitive activity. Rats were tested on a 4-arm spontaneous alternation task, immediately, 1 hr, or 24 hr before learning a hippocampus-sensitive place task or a striatum-sensitive response task. Spontaneous alternation testing 1 hr prior to training enhanced learning of both tasks. Rats tested on a straight runway version of the spontaneous alternation maze, however, did not show enhancements in place and response learning, suggesting that the cognitive load and not physical activity on the maze used for priming underlies enhancements of later learning. The effect of cognitive priming on concentrations of mature brain-derived neurotrophic factor (mBDNF) in the hippocampus and striatum was examined immediately, 1 hr, or 24 hr following 4-arm spontaneous alternation testing. mBDNF in both the hippocampus and striatum increased within 1 hr after alternation testing. K252a, a drug that blocks BDNF-TrkB signaling, or vehicle was infused into the brain 30 min prior to place or response training. K252a infusions into the hippocampus and striatum attenuated the enhancements in place and response learning, respectively, induced by prior alternation testing; K252a treatment did not impair learning in control rats. These results show that,

like physical activity, cognitive activity can also enhance learning of both hippocampus- and striatum-sensitive tasks through BDNF-TrkB signaling.

INTRODUCTION

Environmental enrichment (EE), a stimulating environment that includes novel objects/toys, nesting material, tunnels, ladders, running wheels, and social interactions has been shown to enhance multiple domains of cognition (Greenough et al., 1969; Krech et al., 1962; van Praag et al., 2000). In addition, EE affects brain structure and chemistry (Mora et al., 2007; Bennett et al., 1964; Landers et al., 2011; Mychasiuk et al., 2014; Moser et al., 1994; Nilsson et al., 1999; Leggio et al., 2005; Volkmar et al., 1972; Diamond et al., 1972; Black et al., 1989; Kleim et al., 1997; Saito et al., 1994). Given that voluntary exercise is such a large component to the EE paradigm, it has been hypothesized that the cognition-enhancing benefits of EE are mediated through voluntary exercise (Bekinschtein et al., 2011; van Praag et al., 2008).

In addition to the component of EE contributed by physical activity, there is also a major cognitive component derived from social interactions with conspecifics and exploration and manipulation of objects in the arena. The influences of cognitive experiences on learning and memory have received relatively less attention than have the physical components of these experiences. Cognitive training enhances performance on later cognitive tasks in young adults (Olesen et al., 2004; Dahlin et al., 2008; Owen et al., 2010; Schmeidek et al., 2010; Studenski et al., 2006; Rosenzweig and Bennett, 1996; Light et al., 2010) and the elderly (Basak et al., 2008; Hall et al., 2009; Anguera et al., 2013; Mora et al., 2007; Petrosini et al., 2009).

Recently, we found that voluntary exercise results in enhancement of both place and response learning (Chapter 3; Korol et al., 2013). In addition, we found that mature brain-derived neurotrophic factor (mBDNF) signaling during learning appears to mediate the exercise-induced enhancement of learning in both tasks. Given that cognitive activity also increases BDNF expression and protein in the areas activated by task-engagement (Kim et al., 2012; Kesslak et al., 1998) we hypothesize that, like physical activity, the cognitive benefits afforded by cognitive activity are likely a consequence of heightened BDNF signaling at the time of learning. BDNF and its cognate receptor, TrkB, are found in both the striatum and hippocampus (Maisonpierre et al., 1990; Phillips et al., 1990; Yan et al., 1997; Croll et al., 1998; for review see Zuccato and Cattaneo, 2009). In addition, BDNF and TrkB expression in the hippocampus and striatum correlate with cognitive abilities (Croll et al., 1998; Vaynman et al., 2004).

The current study uses an approach similar to what we applied to studying the effects of physical activity on cognitive functions (Chapter 3). Here, a spontaneous alternation task was used as a behavioral primer before rats were trained on a hippocampus-sensitive place task or a striatum-sensitive response task. An inhibitor to TrkB, K252a, was infused into the hippocampus and striatum of controls and cognitively primed rats to test if, similar to physical activity, cognitive activity enhances learning via BDNF-TrkB signaling.

METHODS

Subjects

Three-month-old, male Sprague-Dawley rats were obtained from Harlan Laboratories (Oregon, Wisconsin, USA). Upon arrival, the rats were housed singly in

clear plastic cages (48 cm L x 26 cm W x 21 cm H) with free access to water and were kept on 12/12 hr light/dark cycle. Rats also had free access to food upon arrival until a food restriction regimen was begun.

Seven days before behavioral testing, rats were placed on food restriction to reduce their body weights to 80-85% of their free-feeding weight. Rats were either behaviorally primed with spontaneous alternation testing or left undisturbed in their home cage until being trained on place or response learning tasks. In Experiments 1, 2, and 4, primed rats were later trained on place and response learning tasks 5 min (immediate), 1 hr, or 24 hr after priming. Behaviorally naïve, untested control rats were trained at similar times in the day. A subset of control and primed rats were not trained in place and response learning tasks (Experiment 3). Instead, their brains were harvested for assay of mBDNF either 5 min (immediate), 1 hr, or 24 hr after priming or at the same time of the day for controls.

Experiments:

Schematics for the experimental protocols followed in the four experiments are illustrated in Figure 1. Different sets of rats were used for each experiment. Experiment 1 tested the effects of cognitive priming with a spontaneous alternation task that engages spatial working memory. Experiment 2 dissociated the role of cognitive activity from locomotor activity during priming with spontaneous alternation testing using a maze with a relatively low cognitive load but similar movement requirements. Experiment 3 measured tissue content of mBDNF in the hippocampus and striatum after spontaneous alternation testing. Experiment 4 determined whether BDNF-TrkB signaling during place or response learning was needed to observe the priming effect.

Spontaneous Alternation Maze Testing

In Experiments 1, 3, and 4, rats primed with spontaneous alternation were placed on a 4-arm, plus-shaped maze (Figure 2A). The maze was located in the center of the 3.5 m x 3 m testing room on a table 76 cm above the floor surrounded by a rich assortment of extra-maze visual cues. During a testing session, the rat was placed in a start arm and allowed to explore the maze freely for 20 min while the number and sequence of arm entries were recorded. An alternation occurred when the rat entered all four arms within 5 consecutive arm entries. On average, rats entered 32 arms per SA testing session. A moving window of 5 arm entries was analyzed for possible alternations across all arms entered by the rat within a single testing period. For example, if a rat entered arms ABDAC in 5 consecutive arm choices; this would be considered an alternation. However, if the rat entered arms ABACB in 5 consecutive arm choices; this would not be considered an alternation.

Rats in Experiment 2 were treated the same but were tested on a 2-arm straight alley maze (Figure 2B) to reduce the cognitive load of the 4-arm maze in Experiment 1 while retaining similar locomotor activity.

Place and Response Learning tasks and Maze Environment

Place and response training were conducted in a 4-arm, plus-shaped maze configured into a “T” in which each trial started from either the north or south arms. For both tasks, the rats were trained to locate a food reward (half of a Frosted Cheerio ©) at the end of an arm. Training took place between 3 and 8 hr after the start of a light cycle.

The full length of each alleyway of the T-maze was approximately 104 cm. Each of the four arms of the maze was 13 cm wide, 18 cm high, and 46 cm in length. The center of the maze, from which all four arms extended, was 12 cm². The maze walls were constructed of black, 0.64-cm-thick Plexiglas® and the floor of black, matte Plexiglas®. The maze was affixed to a platform (46 cm in diameter) that allowed rotation of the maze. Plastic perforated receptacles with inaccessible food were attached to the ends of each maze arm to discourage the use of odor cues to find the reward. A lamp with indirect lighting was placed in each corner of the room, providing symmetrical ambient light. A fan was used to mask building noise.

Training Procedure

In the place task, rats learned the location of a food reward relative to the extramaze cues in the room (Figure 2C). Rats trained on the response task learned to locate a food reward using specific body turns (i.e. right or left turns; Figure 2D). In the response task, ceiling-to-floor curtains surrounded the maze to minimize extramaze cues. The presence or absence of extramaze cues promotes the use of place or response strategies for efficient learning. However, the two versions of the task have the same locomotor and motivational requirements. For each rat, start arms were quasi-randomized across twenty trials, so that each start arm (north or south) was used for 10 trials. For the place task, the goal location (east or west) was counterbalanced while for the response task the direction of body turn (right or left) was counterbalanced across rats within a treatment condition.

Rats were placed in a fresh, clean cage without food or water and moved into the training room for 15 minutes to allow acclimation to the maze environment prior to training; rats in the immediate group were allowed a 5-min acclimation period.

A trial started when a rat was placed in the start arm and ended after all four paws were placed in a choice arm. For each trial, the rat was given the choice of entering one of the two possible reward arms. When the rat chose the rewarded arm, it was allowed to remain there until eating was complete before being returned to the holding cage for 30 sec before the next trial began. If the rat chose the incorrect arm, it was allowed to remain in that arm for approximately 5 sec before being returned to the holding cage for 30 sec before the next trial. The maximum time allotted for rats to choose an arm was 2 min, after which the rat was returned to the cage without making an arm choice. These omissions were not considered arm choices and did not contribute to the number of recorded correct or incorrect choices. Between trials, the maze was rotated 90° to reduce the use of intramaze cues to find the goal arm.

All rats received 75 training trials. The learning criterion was 9 correct trials out of 10 with at least 6 consecutive correct trials. Immediately after training (Experiments 1, 2, 4) or spontaneous alternation testing (Experiment 3), rats were overdosed with sodium pentobarbital (75 mg/kg), after which they were decapitated and brain tissue was collected for histological evaluation of cannula placements or biochemical assessment. Control rats were not tested on the spontaneous alternation task and were euthanized at times similar to those of rats tested on the alternation task.

Determination of brain mBDNF concentrations

Rats were euthanized by overdose with sodium barbitol (75 mg/kg) and decapitated. Brains were removed and dissected on ice and within 150 sec of decapitation, and tissue flash frozen on dry ice and stored at -80°C. The samples were later homogenized on ice in a lysis buffer (50 mmol/L tricine, 0.1% β -mercaptoethanol, 10 mmol/L EDTA, 100 mmol/L NaF, 0.5 mmol/L PMSF and 1 L dH₂O) and dissolved protease inhibitor tablets (Roche Diagnostic Corporation, Indianapolis, IN). The samples were then transferred to a microfuge tube and centrifuged at 4°C for 15 min at 10,000 rpm. The supernatant was collected and stored at -20°C until assayed for protein and mBDNF content.

The protein concentrations of the samples were determined using a Micro-BCA assay kit (Pierce, Rockford, IL). Each tissue sample was diluted 1:16 with the lysis buffer. Standards (25 μ l) ranging from 0 μ g/ml to 2000 μ g/ml and diluted samples (25 μ l) were added in triplicate to a 96-well microtiter plate. A working reagent (100 μ l) was added to each well and incubated at 37°C for 30 min. After incubation, the plate was cooled to room temperature (21°C). Absorbance at 570 nm was then measured in a ThermoMax® microplate reader with Ascent software (Fisher Scientific, Pittsburgh, PA).

BDNF was measured in hippocampus and striatum samples.

Sandwich-style enzyme-linked immunosorbent assays (ELISAs) were performed using the BDNF Emax ImmunoAssay System kit (Promega, Madison, WI) according to manufacturer's instructions to detect mBDNF. The absorbance was read at a wavelength of 450 nm (within 30 min of stopping the reaction) in a ThermoMax microplate reader with Ascent software (Fischer Scientific, Pittsburgh, PA). mBDNF

content was interpolated from standard curves conducted on each plate (0–500 pg/ml for BDNF). To adjust for differences in the total levels of protein across samples, the mBDNF protein value was divided by the total sample protein value to determine mBDNF per unit protein (pg/mg).

To determine the recovery rates of mBDNF for our protocol, a spike and recovery assay was performed on the hippocampus and striatum samples. Spiked samples were compared to unspiked samples and the percent recovery was calculated.

Cannula implantation

In a subset of rats (Experiment 4), cannulae were implanted bilaterally into either the hippocampus or striatum at least three days after arrival and before any experimental procedures. Rats were anesthetized with 2-3.5% isoflurane and received penicillin (30,000 units, i.m.; Dura-Pen; Henry Schein, Inc., Indianapolis, IN, USA) and Rimadyl (5 mg/kg, s.c.) prior to aseptic stereotaxic surgery. Rats were secured in the stereotaxic device with the skull at 180° angle. The skull was exposed through an incision in the scalp and cleared of the fascia before burr-holes were drilled for cannulae and skull screws. Stainless steel guide cannulae (22 gauge, 6 mm; Plastics One, Inc., Roanoke, VA, USA) were lowered into the dorsal hippocampus (AP -3.8, ML \pm 2.5, -1.9 mm ventral to dura) or the dorsal striatum (AP +0.2, \pm ML 3.6, -2.8 mm ventral to dura). Coordinates were chosen based on reports demonstrating drug and hormone actions in the hippocampus and striatum on place and response learning (McElroy and Korol, 2005; Zurkovsky et al., 2006, 2007, 2011; Newman et al., 2011) based on the Paxinos and Watson (1986) atlas. Four stainless steel screws placed into the skull served as anchors for the assemblage that was cemented in place with dental acrylic. To keep

cannulae open, 28 gauge stylets (Plastics One, Inc.) cut to the length of the guide cannulae were inserted at the time of surgery and were removed only during the central infusions. Rats were allowed to recover from surgery for at least one week before food restriction procedures were initiated.

Central infusions

Rats with cannulae received bilateral infusions of K252a (25 μ M in vehicle) or vehicle alone (5% DMSO in aCSF in mM: 128 NaCl, 2.5 KCl, 1.3 CaCl₂, 2.1 MgCl₂, 0.9 NaH₂PO₄, 2.0 Na₂HPO₄, 1.0 dextrose) into either the hippocampus for rats trained on the place task or the striatum for rats trained on the response task. A CMA microdialysis infusion pump was used to deliver the K252a or vehicle through a 29-gauge needle attached to a 10 μ l Hamilton syringe at a volume and rate of 0.5 μ l in 1 min. The needle was left in place for 1 min to allow for diffusion away from the infusion site. Infusions were made 30 min after priming with spontaneous alternation and 30 min prior to training on the place or response learning task.

Experimental Designs:

Experiment 1. Rats were randomly assigned to spontaneous alternation testing or control conditions. Rats were tested first for 20 min on a 4-arm spontaneous alternation maze. These rats were subsequently trained on either a place or a response task immediately (place: N=8; response: N=7), 1 hr (place: N=7; response N=5), or 24 hr (place: N=7; response: N=8) following priming with spontaneous alternation testing. Control rats were trained on the place (immediate N=10; 1 hr N=8; 24 hr N=9) or response (immediate N=9; 1 hr N=5; 24 hr N=8) tasks without prior alternation testing.

Experiment 2. We previously found that cognitive load of a spontaneous alternation task is a function of the number of open arms (McNay et al., 2000; Korol, 2002). In this experiment we attempted to reduce cognitive load but match the locomotor activity level from Experiment 1 to assess whether motor activity was driving the observed learning enhancements. Rats were allowed to explore the straight alleyway maze for 20 minutes, moving freely through the two open arms. Rats were subsequently trained on the place (N=5 runway; N=4 control) or response (N=4 runway; N=4 control) task 1 hr following alternation testing in this straight-arm maze. Except for the difference in the maze used for priming, all other procedures were the same as in Experiment 1.

Experiment 3. Rats were placed on a 4-arm maze for spontaneous alternation testing and were sacrificed immediately (N=6), 1 hr (N=6), or 24 hr (N=5) later. A subset of rats was food restricted for 7 days but never tested on the spontaneous alternation maze and used for control comparisons (N=6). Rats were anesthetized with a lethal injection of sodium pentobarbital (i.p. 75 mg/kg) and brains harvested for assay of mBDNF.

Experiment 4: One week after surgery for cannula implantation, rats were placed on food restriction for 5-7 days until they reached 80%-85% free feeding body weight. Rats were then primed with spontaneous alternation testing or served as controls. Intrahippocampal or intrastriatal infusions of K252a or vehicle [(artificial cerebrospinal fluid (aCSF) and 5% dimethyl sulfoxide (DMSO)] were made 30 min after spontaneous alternation testing and 30 min prior to place or response learning. This created four experimental groups for each of the learning tasks: 1) rats with prior spontaneous

alternation testing given vehicle (N=8 for place, N=8 for response learning), 2) rats with spontaneous alternation testing given K252a (N=7 for place, N=6 for response learning), 3) control rats given vehicle (N=10 for place, N=8 for response learning) and 4) control rats given K252a (N=7 for place, N=7 for response learning).

Following training, rats were given lethal injections of sodium pentobarbital (i.p. 75 mg/kg). The brains were checked for proper cannula placement in the hippocampus and striatum. Specifically, the criteria for misplaced cannula included if cannula were not bilaterally placed in the structure of interest (i.e. cannula tracts were: too dorsal (tracts found in cortex and not penetrating the structure of interest), too ventral (in the case of hippocampus implants tracts found in the thalamus), too medial (in the case of the striatum implants, tracts found in or very near ventricles), or too lateral (in the case of the hippocampus, tracts found in cortex, lateral to hippocampus). Rats with misplaced cannula (N=4), or damage beyond the cannula site (i.e large lesion surrounding brain region) (N=1) were discarded from analysis.

Data analysis and statistics

Confidence intervals (95%) were used to test if the mean SA scores in Experiments 1, 3, and 4 fell above the chance behavior score of 44%.

Experiment 1: Because the control rats showed variability in trials to reach criterion (9/10) across different time points (i.e. immediate, 1 hr, 24 hr), trials to criterion data for the place and response tasks are presented as percent of controls for each set of rats. One-way analyses of variance (ANOVA) were used to analyze the effects of prior spontaneous alternation testing on trials to reach criterion (9/10) in the place and response tasks ($\alpha=0.05$).

Experiment 2: The effect of straight runway testing on trials to reach criterion 9/10) in place and response learning was analyzed using two-tailed Student's *t*-tests as in Experiment 1. The comparison between arm entries in the 4-arm and the straight runway SA testing was analyzed using two-tailed Student's *t*-tests.

Experiment 3: The effects of spontaneous alternation testing on subsequent mBDNF content in the hippocampus, striatum, and hippocampus:striatum were analyzed using two-way ANOVAs to test for effects of structure (hippocampus and striatum) and time following SA testing (immediate, 1 hr, 24 hr) .

Experiment 4: Because not all rats in Experiment 4 learned the place and response tasks within the 75 trials allotted, the distribution of scores were truncated. Therefore, non-parametric two-tailed Mann-Whitney U-tests were performed to compare the effects of cognitive priming and drug treatment.

All procedures followed the institutional and federal guidelines and were approved by the IACUC at the University of Illinois.

RESULTS

In all experiments that used 4-arm SA testing as a behavioral primer, rats had similar levels of alternation. In all experiments the rats had alternation scores well above the 44% chance behavior (Figure 3.3A). In Experiment 1, place-trained rats alternated with a mean score of 61, CI 56.6-65.4. Response-trained rats alternated with a mean score of 67, CI 61.4-72.5. In Experiment 3 rats alternated with a mean score of 64.2, CI 56.9-71.4. In Experiment 4 place-trained rats alternated with a mean score of 59, CI 49.9-68. Response-trained rats alternated with a mean score of 56, CI 48.3-63.6. In addition, in all experiments the rats entered a similar numbers of arms during the SA

testing session (Figure 3.3B).

Experiment 1: Priming with spontaneous alternation testing resulted in an overall enhancement of learning on both the place and response tasks (Figure 3.4). There was a main effect of priming with SA testing on place training ($F_{(3,45)}=4.6$, $p<0.01$; Figure 3.4). Post hoc analyses revealed significantly lower trials to criterion were evident for place learning (Figure 3.4, left panel) in rats trained either 1 hr ($t_{13}=2.47$, $p<0.05$) or immediately ($t_{14}=2.2$, $p<0.05$) after spontaneous alternation testing; rats with a 24-hr delay after spontaneous alternation testing exhibited mean trials to criterion similar to those rats tested at shorter intervals but this difference did not reach statistical significance ($t_{14}=1.71$, $p\leq 0.1$). There was no significant main effect of priming with SA testing on response learning ($F_{(3,38)}=2$, $p\leq 0.1$; Figure 3.4). Rats trained on the response task (Figure 3.4, right panel) took significantly fewer trials to reach criterion in the 1-hr condition ($t_8=2.2$, $p\leq 0.05$) but not after the 24-hr ($t_{13}=0.88$, $p>0.2$) or immediate ($t_{15}=1.5$, $p>0.1$) delays, though those mean trials to criterion were also in the direction of enhancement. Because learning enhancements in both tasks were robust one hour after spontaneous alternation testing, that time between priming and tests of learning was used for Experiments 2, 3, and 4.

Experiment 2: Priming on the straight runway 1 hr prior to testing did not enhance later learning in either place or response tasks. Compared to controls, testing in the straight runway maze had no significant effect on the number of trials to reach criterion in the place ($t_7=0.33$, $p>0.2$; Figure 3.5A) or response ($t_6=0.07$, $p>0.2$; Figure 3.5B) tasks. Of note, the number of arms entered on the straight runway maze was comparable to arms entered in Experiment 1 on the four-arm maze ($t_{18}=0.35$, $p>0.2$;

Figure 3. 5C), indicating similar locomotor activity on the two mazes.

Experiment 3: mBDNF content in the hippocampus and striatum of rats exposed to four-arm spontaneous alternation testing but not trained subsequently on place or response learning tasks showed changes over the 24-hr period following testing. There was no significant main effect of time following SA testing on mBDNF levels in the hippocampus ($F_{(3,18)}=2.1$, $p \leq 0.1$; Figure 3.6A), but there was a significant main effect of time following SA testing on mBDNF levels in the striatum ($F_{(3,19)}=7.2$, $p < 0.01$; Figure 3.6B). Immediately after spontaneous alternation testing, mBDNF concentrations were unchanged in the hippocampus ($t_{12}=0.49$, $p > 0.2$) and striatum ($t_{12}=0.23$, $p > 0.2$) compared to values from control rats that remained in their home cages. Both hippocampal ($t_9=2.2$, $p \leq 0.05$; Figure 3.6A) and striatal ($t_{10}=2.2$, $p \leq 0.05$; Figure 3.6B) mBDNF concentrations were significantly increased at 1 hr following spontaneous alternation testing compared to those of controls. However, the change in mBDNF concentrations from 1 hr to 24 hr after priming was remarkably different for the hippocampus and striatum. For the hippocampus, mBDNF concentrations fell to baseline levels at 24 hr ($t_9=0.53$, $p > 0.2$; Figure 3.6A). For the striatum, mBDNF concentrations increased significantly further from 1hr to 24 hr. The 24-hr values were significantly higher than mBDNF concentrations in control rats ($t_9=3.00$, $p \leq 0.01$; Figure 3.6B), and were also higher, but not significantly than those seen 1 hr after spontaneous alternation testing ($t_9=1.9$, $p \leq 0.1$). Two-way ANOVAs revealed a main effect of brain region ($F_{(1,37)}=27.9$, $p < 0.0001$), with the mBDNF levels in the hippocampus (Figure 3.6A) being significantly higher than the levels in the striatum (Figure 3.6B). There was no main effect of time point ($F_{(3,37)}=2.7$, $p > 0.2$) on mBDNF levels. There was a

significant interaction between brain region and timepoint ($F_{(3,37)}=4$, $p \leq 0.01$).

Experiment 4: Infusions of the TrkB inhibitor K252a, into the hippocampus or striatum blocked the enhancements of place and response learning seen 1 hr after priming with the alternation task. The priming-related enhancements demonstrated in Experiment 1 were replicated here, as seen by decreased trials to criterion in rats that were primed and that received vehicle control infusions compared to vehicle-treated control rats (place: $U_{9,10}=5.5$, $p < 0.05$; Figure 3.7A, $p < 0.05$; response: $U_{8,8}=15$, $p < 0.05$; Figure 3.7B, $p < 0.05$) (SA-Veh vs control-Veh; Figure 3.7). K252a injections in the primed rats fully blocked the enhancements on both tasks. Rats that were primed with spontaneous alternation and treated with K252a into the hippocampus required significantly more trials to learn the place task than did primed rats treated with vehicle (SA-K252a vs SA-Veh, $U_{9,8}=0.5$, $p < 0.01$; Figure 3.7A). Similarly, primed rats with infusions of K252a into the striatum required significantly more trials to reach criterion than did vehicle-treated primed rats (SA-K252a vs SA-Veh, $U_{6,8}=0$, $p < 0.01$; Figure 3.7B). On both tasks, the K252a injections resulted in scores in the primed rats that were comparable to those of the control rats with no prior alternation testing (SA-K252a vs. control-Veh) for either the place ($U_{10,7}=19$, $p > 0.05$; Figure 3.7A) or response ($U_{7,8}=15.5$, $p > 0.05$; Figure 3.7B) tasks.

Importantly, treatment with K252a in unprimed rats failed to impair learning in either task (control-K252a vs control-Veh). Specifically, K252a infusions into the hippocampus of control, unprimed rats had no effects on learning in the place ($U_{7,10}=19$, $p > 0.05$; Figure 3.7A) or response task ($U_{7,8}=15$, $p > 0.05$; Figure 3.7B).

DISCUSSION

The present findings show that a single bout of cognitive activity, provided by 20 min of testing on a spatial working memory task, enhances place and response learning in rats. Furthermore, the results indicate that blockade of BDNF-TrkB signaling during learning, blocks the anterograde benefits accrued from the prior alternation testing during later place and response training. The place and response tasks are, respectively, sensitive to manipulations of the hippocampus and striatum. Therefore, it is likely that cognitive activity with spontaneous alternation testing enhances learning mediated by processing in multiple memory systems.

Prior findings also indicate that prior spatial memory testing can enhance learning on later navigation tasks requiring spatial or egocentric learning (Klingberg et al., 2002). More recently, twelve consecutive days of working memory training in an 8-arm radial maze was shown to enhance learning in a variety of tasks including odor discrimination, passive avoidance, associative fear conditioning, egocentric navigation in a Lashley maze, and spatial navigation in the swim task (Light et al., 2010). These reports are part of a larger set of findings showing that enriched environments, which combine elements of cognitive activity, physical exercise, and some times social interactions, enhance later learning (Leggio et al., 2005; Toscano et al., 2006; Hoplight et al., 2001; Tees, 1999; Lonetti et al., 2010). The enriched environment procedures result in reorganization of dendritic spines and vascular measures in some brain areas (Black et al., 1987; Sirevaag et al., 1988; Cao et al., 2004; Leggio et al., 2005; Volkmar et al., 1972); however, the relationship to BDNF signaling during the time of cognitive testing is unknown.

Cognitive load of the priming may play an important role in determining which experiences will enhance subsequent cognition. In our study, a 20-minute test in the two-arm, straight alley maze was not sufficient to enhance later learning on either the place or response task. Of note, the number of arm entries during 20-min tests in the runway and four-arm alternation tasks was comparable, indicating that locomotor activity per se was not a key variable contributing to enhancement of learning. Instead, it appears that the cognitive load, i.e. in a 2- vs. 4-arm maze, was important for these effects. We have found previously that decreasing the cognitive load in the same manner, by decreasing the number of arms in the maze, reduces the magnitude of depletion of extracellular glucose levels during alternation testing (McNay et al., 2000). Therefore, a possibility to be addressed in the future is whether prior metabolic demand in the hippocampus is important for enhancement of later learning. Parallel information is not available regarding the effects of memory testing on striatal glucose levels during testing on a working memory task.

A parallel experiment examined mBDNF tissue concentrations immediately, 1 hr, and 24 hr after 20-min tests of spontaneous alternation in the 4-arm maze. The mBDNF concentrations in both hippocampus and striatum increased after spontaneous alternation testing. The increases were significant in both brain areas at 1 hr after testing and in the striatum remained higher than those of controls even 24 hr after priming. Recently, we found that extracellular BDNF in the striatum continues to increase beyond baseline levels following learning a striatum sensitive task, whereas extracellular BDNF in the hippocampus returns to baseline following learning a hippocampus sensitive task (Chapter 4). mBDNF levels varied by brain area, with

higher control values seen in the hippocampus than in the striatum. The regional variations in mBDNF levels are consistent with past reports of total BDNF in the hippocampus and striatum (Chapter 4; Conner et al., 1997). For example, in the following chapter, we found that extracellular BDNF levels are higher in hippocampus than in the striatum.

BDNF is an activity-dependent protein that increases its synthesis and release in response to cellular excitation (Zafra et al., 1990; Patterson et al., 1992; Hartmann et al., 2001; Lu, 2003). Importantly, mBDNF protein and mRNA content increase immediately following a learning event (Kesslak et al., 1998; Hall et al., 2000; Kim et al., 2012) and remain raised for several hours following (Kim et al., 2012). In the present experiment, mBDNF protein content did not increase immediately after testing on the working memory task but did increase 1 hr later. It is possible, then, that the induction of mBDNF content in both hippocampus and striatum 1 hr after alternation testing provided increased signaling that mediated the enhancement of learning on both the place and response tasks.

The striking increase in striatal mBDNF concentration in the striatum 24 hr after alternation testing was not associated with enhanced response learning at that time. It is important to note that the tissue measurements of mBDNF in the current study reflect both intra- and extra-cellular levels of mBDNF. Increased tissue levels of mBDNF might reflect increased availability for release and signaling, but the increases might also reflect increased intracellular levels as a result of lowered mBDNF release. Currently, our lab is performing studies to investigate if alternation testing leads to an increase in mBDNF release during place and response training.

At the 1-hr time point, mBDNF tissue levels were elevated after cognitive priming and learning was enhanced in both tasks. The pharmacological results obtained with the TrkB receptor antagonist, K252a, are consistent with the interpretation that mBDNF signaling is important for enhancement of learning at the 1-hr time point. In these experiments, treatment with the TrkB receptor antagonist blocked the enhancement of learning seen in both the place and response tasks. Thus, mBDNF signaling via its cognate TrkB receptor is necessary for the learning enhancements. Our findings and this interpretation are consistent with other pharmacological experiments in which activation of the TrkB receptors with intraamygdala TrkB agonists near the time of fear conditioning enhanced learning and memory for a fear conditioning task (Andero et al., 2011). In addition, experiments that infused BDNF intracortically enhanced conditioned taste aversion memory (Castillo et al., 2006). And Intrahippocampal administration of recombinant BDNF following weak inhibitory avoidance training enhanced memory retention of the shock, and reversed memory deficits following protein synthesis inhibition (Bekinschtein et al., 2008). Similarly, intrastriatal injections of BDNF enhanced learning of a strategy set-shifting task (D'Amore et al., 2013). Other reports also have similar findings that BDNF-TrkB signaling enhances learning and memory processes (Zeng et al., 2012; Tyler et al., 2002). Specifically, signaling events downstream of BDNF-TrkB signaling such as ERK activation (Bekinschtein et al., 2008), PI3K activation, and rises in intracellular calcium (Minichello et al. 2002) all independently are important for the beneficial effects of BDNF on learning. When viewed in the context of these past reports, it is likely that the increase in mBDNF signaling mediates the enhancement of memory seen with cognitive priming as seen

also in other contexts.

K252a treatment before learning attenuated the place and response learning enhancements in SA-tested rats. These data suggest a common mechanism—rapid BDNF-TrkB signaling— underlying the SA-induced learning enhancements in both the hippocampus and striatum. One, some, or all of the rapid signaling cascades downstream of BDNF-TrkB signaling could promote learning and memory processes (see Korol et al., 2013; Figure 8). Possibilities include increased glutamate release via increased intracellular calcium and activation of ERK, (Kang and Schumann 1995; Almeida et al., 2005; Jovanovic et al., 2010), inactivation of glycogen synthase kinase 3 β (GSK3 β) (Ortega et al., 2010), and increased glucose metabolism via activation of PI3K/Akt (Mattson, 2002) or, likely, some combination of these cascades.

Although cognitive priming has long been touted as a means of enhancing learning and memory, the mechanisms underlying cognitive activity-induced learning enhancements have remained elusive. The involvement of mBDNF signaling in enhancement of learning by cognitive priming is very similar to our recent findings obtained with priming by physical activity (Chapter 2; Korol et al., 2013). In the earlier experiments, voluntary wheel running for 3 weeks prior to place and response training, as used in the present experiments, resulted in enhanced learning for both tasks. In this case too, the enhancement was attenuated by treatment with K252a at the time of training. Thus, it appears that cognitive and physical priming each enhance learning and do so by shared mechanisms (see Figure 3.8).

FIGURES

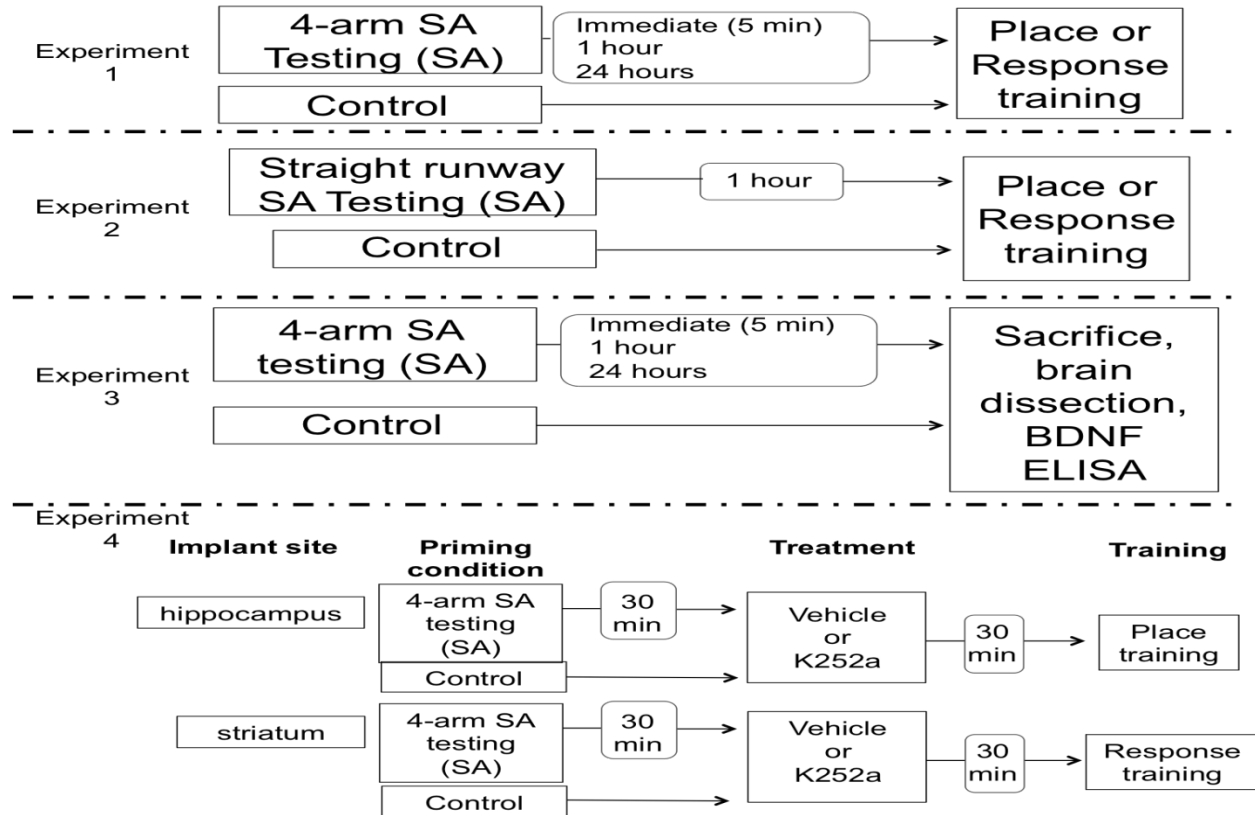


Figure 3.1. Experimental paradigms. In Experiment 1, rats were (SA) or were not (control) tested on the 4-arm SA task immediately, 1 hr, or 24 hr prior to training on the place or response tasks. In Experiment 2, SA rats were tested on a straight runway version of the maze 1 hr prior to training on the place or response tasks. In Experiment 3, rats were SA-tested on the 4-arm maze and killed immediately, 1 hr, or 24 hr following testing. Control rats were sacrificed with no prior behavioral priming. Following sacrifice the hippocampus and striatum were collected for analysis of mBDNF content. In Experiment 4, rats were given bilateral cannula implants in either the dorsal hippocampus or dorsal lateral striatum. Thirty min following SA testing and 30 min prior to place or response training, rats were infused intrahippocampally or intrastriatally, respectively, with K252a or vehicle.

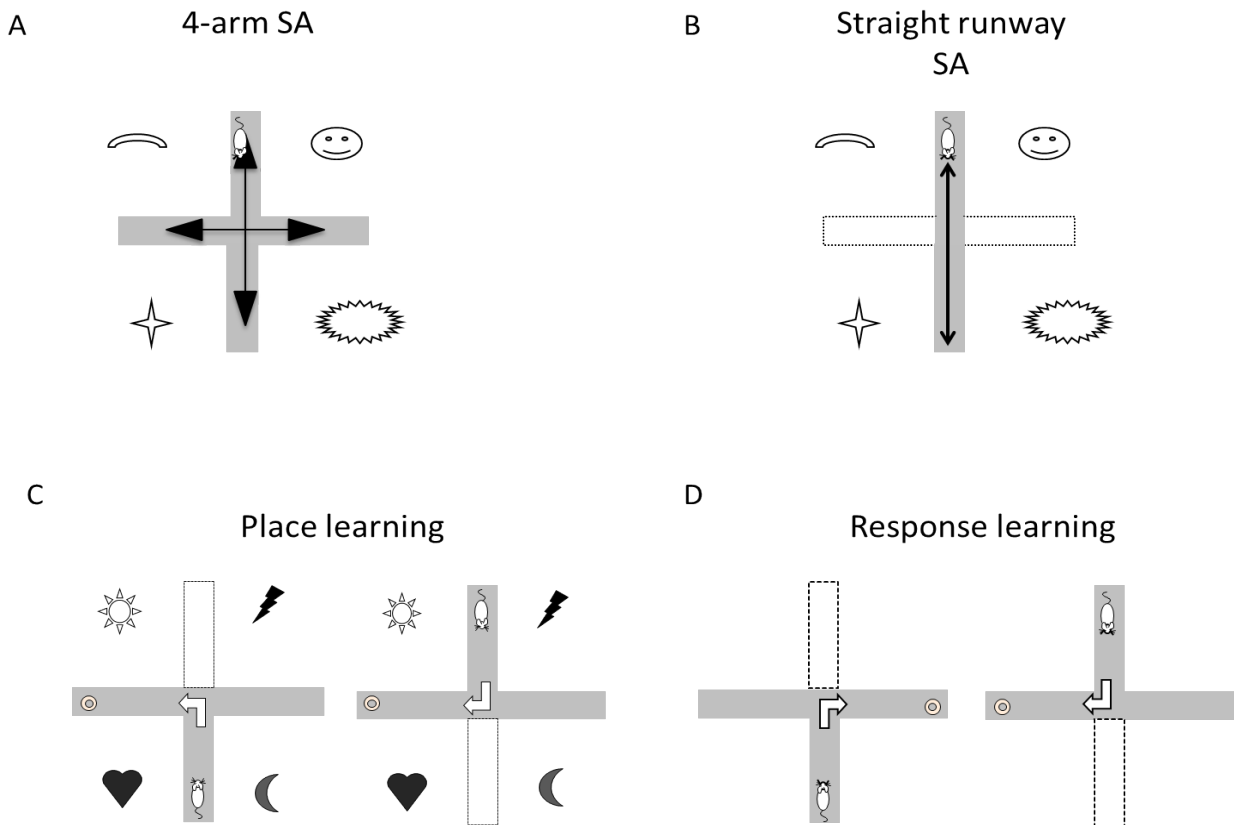


Figure 3.2. Cognitive training and spontaneous alternation testing paradigms. In

A) the 4-arm spontaneous alternation task, rats are given 20 min to explore all 4 arms of the maze and are tested on their working memory capacity by counting how many times the rat enters all 4 arms within 5 arm entries. B) The straight runway spontaneous alternation task is testing a lower cognitive load because the rat can only enter 1 of 2 arms and requires less working memory capacity to successfully explore the available arms of the maze. The place training task C) requires the rat to learn the location of its food reward using the extramaze cues in the room. This task requires intact hippocampal functioning for successful learning. The response training task D) requires the rat to learn the location of its food reward using egocentric body movements (i.e. left or right turns). This task requires intact striatal functioning for successful learning.

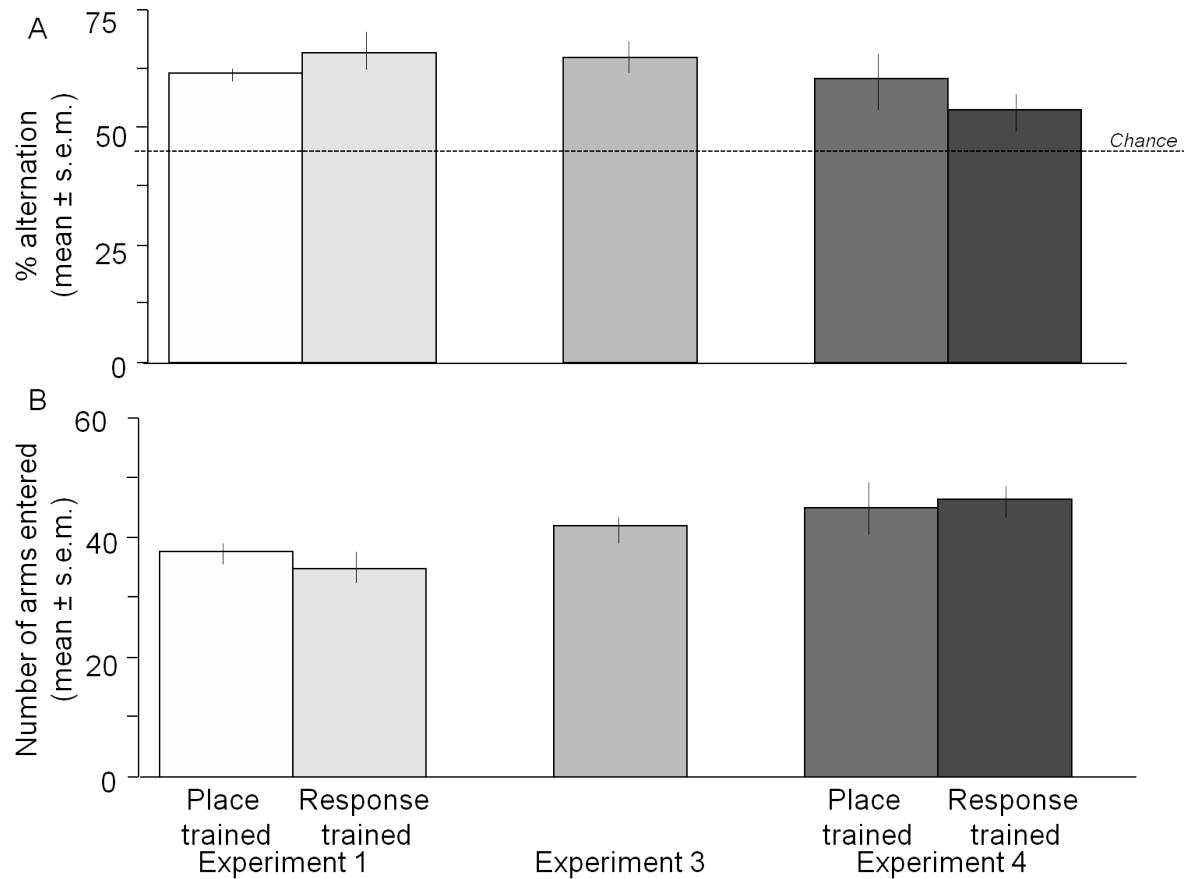


Figure 3.3. Alternation scores and arm entries for SA-tested rats. Rats that were SA tested with the 4-arm maze in Experiments 1, 3 and 4 all had similar alternation scores (A) and arm entries (B). Alternation scores were well above the 44% chance behavior, indicating that all rats that were SA tested with the 4-arm maze were sufficiently engaged in the SA task. For experiment 1 N=22 for place-trained and N=20 for response-trained; for Experiment 3 N=17; for Experiment 4 N=15 for place-trained and N=14 for response-trained.

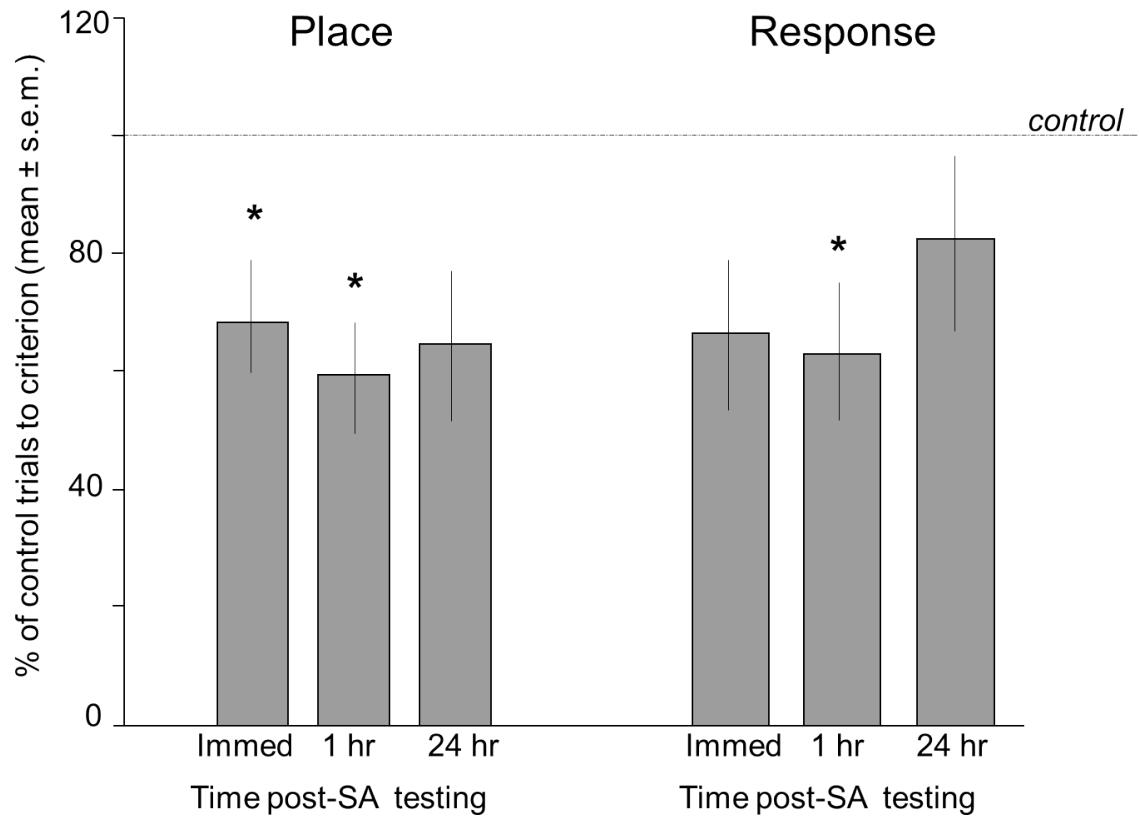


Figure 3.4. Prior 4-arm spontaneous alternation testing enhanced subsequent place and response learning. Prior spontaneous alternation testing enhanced subsequent place learning A) compared to controls, immediately and 1 hr following testing. Response learning B) was enhanced 1 hr following spontaneous alternation testing compared to controls. *= $p \leq 0.05$ vs. untested control. For place training, N=8 for immediate, N=7 for 1 hr and N=7 for 24 hr. For response training, N=7 for immediate, N=5 for 1 hr; N=8 for 24 hr.

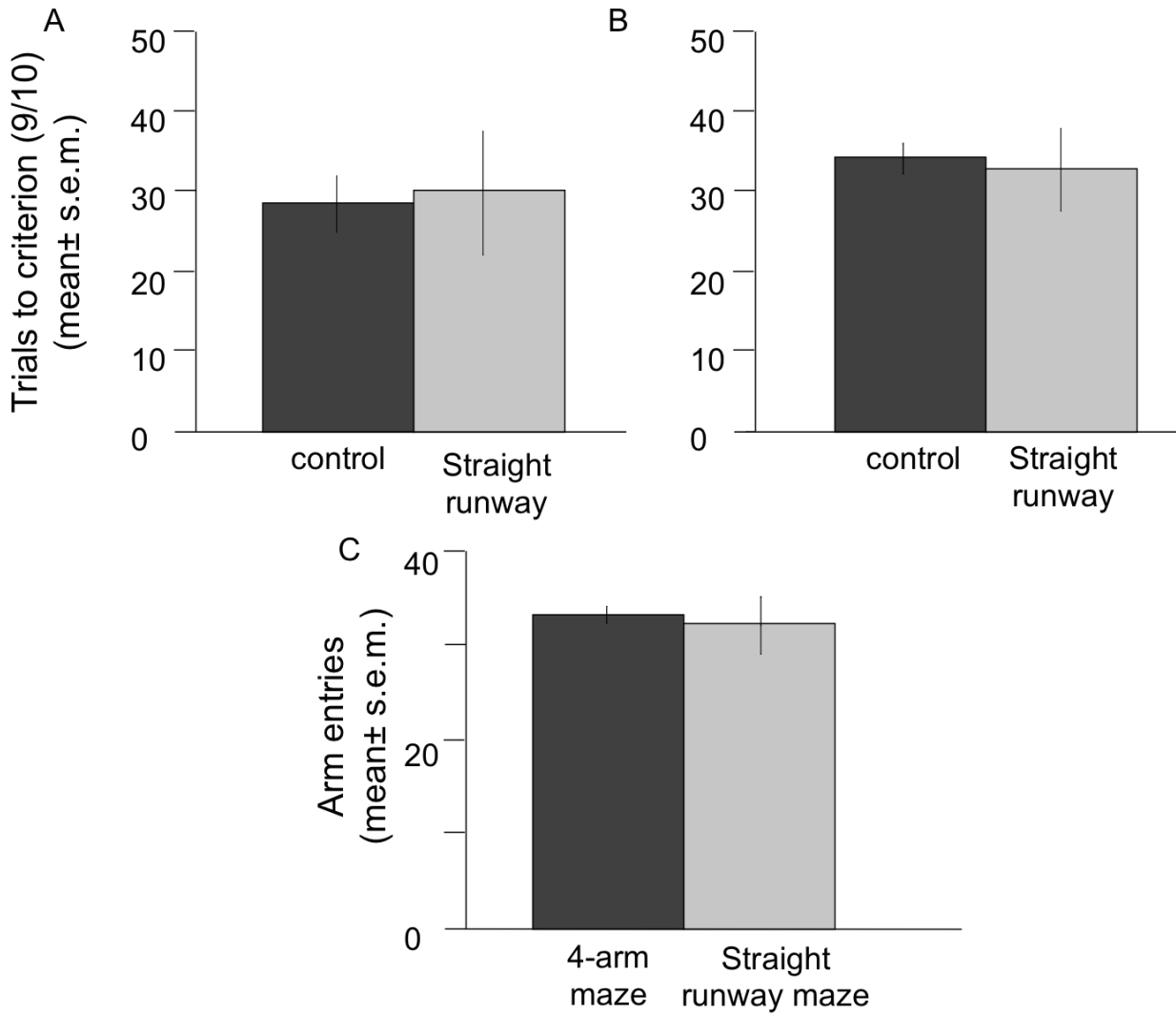


Figure 3.5. Prior straight runway testing had no effect on place or response

learning. Spontaneous alternation rats that engaged in the straight runway maze required a similar number of trials as control rats to reach criterion in the A) place and B) response tasks. In C) arm entries were used to measure locomotor activity on the two versions of the spontaneous alternation maze. Rats that were tested on the straight runway maze entered the same number of arms as rats that were tested on the 4-arm spontaneous alternation maze. For place training N=4 for control, N=5 for straight runway; For response training N=5 for control, N=4 for straight runway.

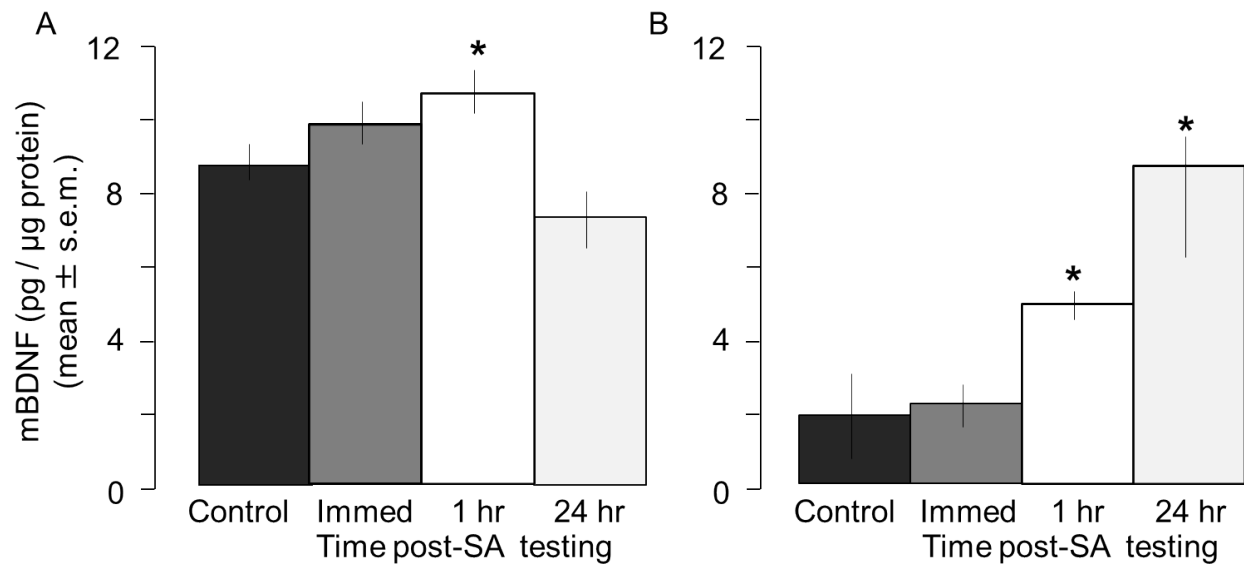


Figure 3.6. 4-arm spontaneous alternation testing increased mBDNF content in the hippocampus and striatum within 1 hr. Within 1 hr following spontaneous alternation testing in the 4-arm maze mBDNF protein was significantly increased in the A) hippocampus and B) striatum compared to control rats. mBDNF content in the B) striatum was significantly increased compared to control rats within 24 hr following spontaneous alternation testing. The hippocampus had significantly higher levels of BDNF compared to the striatum. *= $p \leq 0.05$ vs. controls. N=6 for control; N=6 for immediate; N=5 for 1 hr; N=5 for 24 hr.

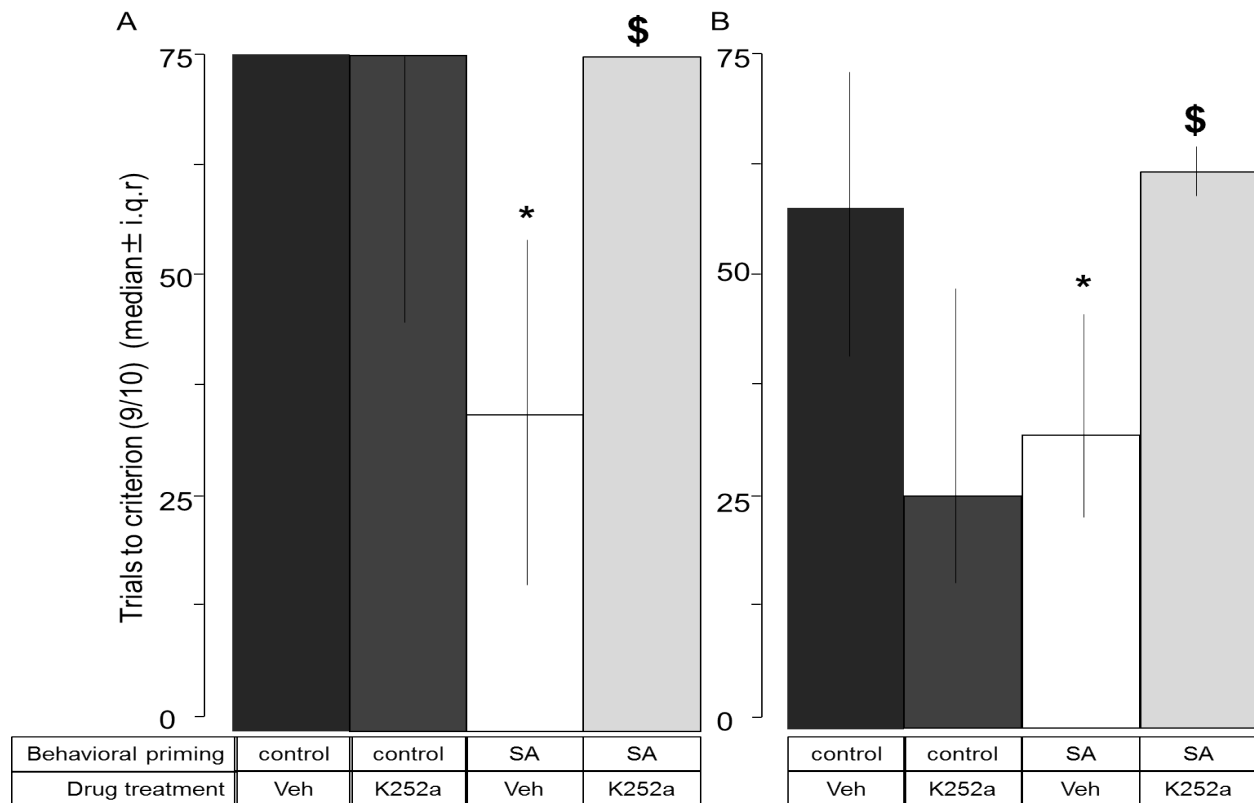


Figure 3.7. K252a treatment attenuates 4-arm spontaneous alternation-induced place and response learning enhancements. K252a treatment in the hippocampus prior to A) place training attenuated spontaneous alternation-induced learning enhancements. Spontaneous alternation-tested vehicle-treated rats learned the place task in fewer trials compared to vehicle-treated control rats. B) K252a treatment in the striatum attenuated spontaneous alternation-induced response learning enhancements. Spontaneous alternation-tested vehicle-treated rats learned the response task in fewer trials than the vehicle-treated control rats. $\ast = p \leq 0.05$ vs. vehicle control; $\$ = p < 0.05$ vs. SA-primed vehicle control. For place-trained rats N=7 for control-Veh; N=7 for control-K252a; N=8 for SA-Veh; N=7 for SA-K252a. For response-trained rats N=8 for control-Veh; N=7 for control-K252a; N=8 for SA-Veh; N=6 for SA-K252a.

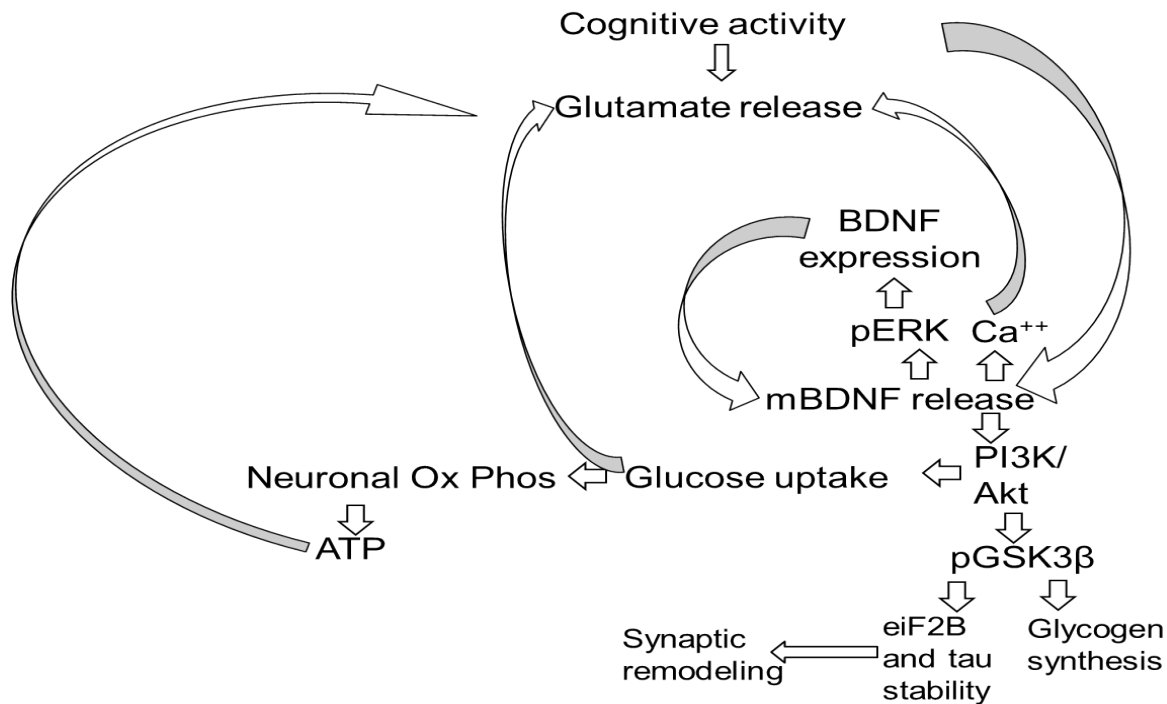


Figure 3.8. BDNF-TrkB signaling induced by use creates a boost for future use.

Cognitive activity induces mBDNF release and signaling. The signaling cascades activated downstream of BDNF-TrkB support ongoing cognitive activity. For example, a rise in intracellular calcium (Ca^{++}) will support neurotransmitter release (i.e. glutamate). Activation of PI3K/Akt signaling inhibits GSK3 β (by phosphorylation at ser9), which leads to glycogen synthesis (supporting future cognitive activity), and allows for protein synthesis (via activation of eiF2B) and stability of tau proteins (allowing for synaptic remodeling). In addition, PI3K/Akt signaling increases glucose uptake, which will provide substrates for oxidative phosphorylation and thus, ATP synthesis. This increase in ATP synthesis further supports neurotransmitter release. Furthermore, glucose itself acts as a precursor for neurotransmitter synthesis. Lastly, activation of ERK (by phosphorylation) signaling initiates gene expression of BDNF, which will lead to increased BDNF availability to support future cognitive activity.

TRANSITIONAL THOUGHTS

Our findings from Chapter 2 and 3 show that cognitive and physical activities enhance place and response learning through BDNF-TrkB signaling. These findings support our theory of “Use it and Boost it”—SA-testing and physical activity engages the hippocampus and striatum, leading to an increased reliance on BDNF-TrkB signaling to support learning in the future. However, several questions remain, why is BDNF-TrkB signaling so important for the learning enhancements in cognitive and physical activity? What is potentiating BDNF-TrkB signaling during learning? In Chapters 2 and 3 we found that SA-testing and 3 weeks of voluntary wheel running increased BDNF protein concentrations in the hippocampus and striatum. Thus, it is likely that cognitively and physically active rats have increased BDNF release in the hippocampus and striatum in response to future neural activity.

Others have shown that BDNF release is activity-dependent; however, no one has ever been able to measure BDNF release in contexts of cognitive and physical activity. In Chapter 4, we investigate how cognitive and physical activities modulate BDNF release by measuring extracellular BDNF in the hippocampus and striatum.

CHAPTER 4:
USING MICRODIALYSIS TO MEASURE THE EFFECTS OF COGNITIVE AND
PHYSICAL ACTIVITY ON EXTRACELLULAR BDNF IN THE HIPPOCAMPUS AND
STRIATUM

ABSTRACT

Previous studies have shown region- and task-specific increases in brain-derived neurotrophic factor (BDNF) tissue content following cognitive testing and physical activity. These measurements encompass both intracellular and extracellular BDNF, making it difficult to discern whether expression, storage, or release of BDNF is the primary target of activity-induced modulation. Given that BDNF is a secreted protein, *in vivo* microdialysis methods were developed and validated via silver stains, Western blots, and enzyme-linked immunosorbent assays (ELISAs) to measure BDNF in the extracellular fluid (ECF). Microdialysis was performed prior to, during, and following cognitive testing in sedentary and physically active rats to detect changes in BDNF release during cognitive activity and modulation of release patterns by prior physical activity. Concentrations of ECF BDNF in the hippocampus and dorsal striatum were measured in sedentary or physically active rats while they were trained on a hippocampus-sensitive place task or a striatum-sensitive response task. Both tasks were conducted on the same 4-arm radial maze using food reward. The microdialysate was collected into micropipette tips (0.5 μ l/ min, 90 min samples) and diluted with sample buffer prior to ELISA quantification of BDNF concentrations. In the hippocampus, place learning produced increases in ECF BDNF from baseline while response training produced decreases. The increase during place learning was potentiated in Exercise rats. The opposite pattern was seen for the striatum, with place training producing decreases in ECF BDNF compared to response training. For the

striatum, exercise did not potentiate the learning induced response in ECF BDNF but instead potentiated baseline levels; this elevated response of ECF BDNF was maintained throughout response training. Because changes in ECF BDNF likely reflect changes in BDNF release, these data show that BDNF release is sensitive to prior physical activity in addition to cognitive demands. Moreover, the striking dissociation in the dynamics of extracellular BDNF during place and response training suggests that region-specific patterns in BDNF release may reflect the cognitive strategies engaged by training in different tasks.

INTRODUCTION

Mature Brain-derived neurotrophic factor (mBDNF) is viewed as a key regulator of neural plasticity associated with various contexts including long-term potentiation of synaptic strength (LTP), learning and memory, drug addiction, and exercise. The acceptance of mBDNF as a component of the cellular signaling involved in plasticity has been established by many approaches including the delivery of antagonists and agonists, genetic manipulations, activation of downstream targets, and analysis of protein and mRNA content (Patterson et al., 2001; Lu, 2003; Tyler et al., 2002; Korte et al., 1996; Patterson et al., 1996; Linnarsson et al., 1997; Korol et al., 2013; Aicardi et al., 2004; Alonso et al., 2002; Andero et al., 2011; Mizuno et al., 2000; Mu et al., 1999). BDNF mRNA and protein content increase following physical activity and cognitive testing in a brain region-specific manner (Kesslak et al., 1998; Ma et al., 2011; Kim et al., 2012; Mizuno et al., 2000; Hall et al., 2000; Cotman and Berchtold, 2002; Gomez Pinilla et al., 2007; Marais et al., 2009; Neeper et al., 1996; Korol et al., 2013). The extracellular cleavage of proBDNF to mBDNF and the subsequent signaling of mBDNF

through its cognate TrkB receptor are potent modulators of learning and memory (for review see Tyler et al., 2002; Lu, 2003; Lu 2005). Furthermore, we have previously shown that increases in BDNF-TrkB signaling in specific memory systems supports enhancements in cognition following priming with exercise (Korol et al., 2013; Chapter 2) and priming with prior cognitive testing (Korol et al., 2013; Chapter 3). Together, these findings point to a specific role for mBDNF signaling in those brain regions selectively engaged during learning and memory.

Though originally considered to act in the retrograde fashion typical of trophic factors, it is now known that BDNF can be released from pre-synaptic elements (Jia et al., 2010; Hartmann et al., 2001) and transported in an anterograde fashion to post-synaptic neurons (Kohara et al., 2001). Converging lines of evidence point to BDNF secretion and signaling as processes that, upon neural activation, increase to induce neural plasticity in activated neural circuits that includes further BDNF expression and release (Zafra et al., 1990; Goodman et al., 1996; Chen et al., 2010; Hall et al., 2000; Patterson et al., 2001; Canossa et al., 1997; Hartmann et al., 2001). Past studies revealing the activity-dependence of BDNF release were conducted *in vitro* using electrophysiological or pharmacological activation or inactivation of neural tissues (Kohara et al., 2001; Balkowiec and Katz, 2002; Greenberg et al., 2009; Naggapan et al., 2009; Kuczewski et al., 2008).

Currently, methods for detecting changes in BDNF as a consequence of, for example, cognitive testing rely on comparisons of tissue content or expression patterns before and after the experimental manipulation and therefore do not directly measure *in vivo* changes during behavior. Moreover, these methods most often use extractions

from whole tissues, comprising vesicular, receptor bound, as well as free extra- and intracellular BDNF, and thus are unable to separate intracellular from extracellular content or distinguish changes in expression, storage, or release.

Because the cognitive benefits afforded by increased mBDNF following exposures to exercise or cognitive priming are attenuated by blocking mBDNF signaling through TrkB receptors, it is likely that the modulation of BDNF release and the cleavage of proBDNF to mBDNF at the time of cognitive testing mediates the enhancements in learning and memory. Furthermore, BDNF release is believed to be an activity-dependent process, though very few *in vivo* studies have directly demonstrated this (Humpel et al., 1995). Thus, techniques in awake behaving animals that allow measures of extracellular BDNF as a proxy for levels of release resulting from cognitive and/or physical activity would create a better understanding of the role that BDNF release and signaling play in learning and memory under various conditions.

In the current study, we developed a novel *in vivo* method of BDNF measurement (that includes both pro and mature BDNF) that combines microdialysis for collection and ELISA for quantification of BDNF in the extracellular fluid (ECF). These techniques were used to test how BDNF release patterns in two different memory systems, the hippocampus and striatum, shift under different cognitive and physical contexts. Importantly, silver stains and western blots were performed to verify, respectively, the range of molecular weights of all proteins and the presence of pro and mature BDNF in the collected dialysate.

All rats were implanted with a microdialysis cannula in the hippocampus or the striatum. ECF samples were collected prior to, during, and following a hippocampus-

sensitive place task or a striatum-sensitive response task. Two approaches were taken in this study. First, to test for dissociations in release of BDNF across these two cognitive tasks, we compared BDNF in the dialysate taken from hippocampus and striatum during place and response learning in sedentary rats. Second, to evaluate the modulatory effects of voluntary exercise on BDNF release and its role in cognition, we compared ECF BDNF in the hippocampus of physically active versus sedentary rats prior to, during, and following place learning. The same comparisons were made for ECF BDNF collected from the striatum in response-trained rats that were physically active or sedentary. Finally, the effects of cognitive priming on BDNF release in the hippocampus and striatum during place and response training were examined. ECF BDNF was measured in the hippocampus or striatum of rats that were SA tested, then place or response trained.

METHODS

Subjects and general procedure

Young adult (90-100 days old) male Sprague-Dawley rats were purchased from Harlan Laboratories (Oregon, Wisconsin, USA). Upon arrival, rats were individually housed in translucent cages with a 12-h light/dark cycle and *ad libitum* access to food (Teklad diet 8604) and water until preparation for cognitive testing. After at least two days acclimation to the vivarium, rats underwent stereotaxic surgery for unilateral guide cannula implantation into the hippocampus or striatum.

Following one week of recovery from surgery, rats were placed in cages with or without voluntary access to running wheels. Rats in the Exercise group were given free access to running wheels for three weeks, while rats in the Sedentary group remained

housed individually in standard cages without wheels. During the week prior to maze training or during the third week of wheel running rats were placed on a food restriction regimen to reduce body weights to 80-85% of their free-feeding weights to motivate learning on both tasks.

On the day of training, a microdialysis probe was carefully inserted into the brain through the guide cannula and dialysis procedures initiated. One hr following the start of microdialysis, baseline sample dialysate was collected for 90 min. Maze training was initiated and the subsequent 90 min sample collection was during place or response learning, and the last 90 min sample collection occurred following maze training when the rat was returned to its home cage. In experiments of ECF BDNF collection in cognitively primed rats, following the 90 min baseline collection, rats were placed on the SA maze for 20 minutes (20 min collection), then returned to home cage and moved to a new room (1 hr collection), then place- or response-trained (90 min collection), and then post training collection was 90 min.

For each brain region sampled, there were three different experimental groups, generating six different groups of rats as follows:

Hippocampus: sedentary, place-trained (n = 7); sedentary, response-trained (n=6); exercise, place-trained (n = 10); cognitively primed, place-trained (n=5).

Striatum: sedentary, place-trained (n = 4); sedentary, response-trained (n = 9); exercise, response-trained (n = 7); cognitively primed, response-trained (n=5).

In experiments of ECF BDNF collection in cognitively primed rats, following the 90 min baseline collection, rats were placed on the SA maze for 20 minutes (20 min collection), then returned to home cage and moved to a new room (1 hr collection), then

place (n=5) or response (n=5) trained (90 min collection), and then post training collection was 90 min.

Cannula Surgery

Two plastic microdialysis guide cannulae (CMA/12 type; 3 mm; Harvard Apparatus, Holliston, MA) were unilaterally implanted under stereotaxic control in rats under isofluorane anesthesia. The cannula was aimed at either the ventral hippocampus (AP=-5 mm; ML=+/-5 mm; DV=-3.2 mm; Figure 4.1A) or lateral striatum (AP=+0.3 mm; ML=+/-4.3 mm; DV=2.7 mm; Figure 4.1B). Four screws were inserted into the skull around the cannula to anchor the cemented head cap on the skull. The assemblage was cemented in place using dental acrylic. All rats were allowed at least one week to recover after surgery.

Running Wheels

All animals in the Exercise group were given access to running wheels (Lafayette Instruments Co.) for 21 days. Running wheels (35.5 cm diameter and 10.9 cm wide) were constructed of stainless steel. The running surface was composed of 1.5 mm rods connected by 7.9 mm centers with a 6.3 mm gap. Transparent plastic walls enclosed the wheels. A rectangular guillotine-style door was built into the wheel and connected via a short stainless steel tunnel to the shoebox cage to restrict or allow access into and out of the wheel. The wheel sat above a steel tray (~40.5 cm L x 36 cm W) to collect debris (Figure 1). In a separate room, a computer recorded running distances by tracking wheel revolutions using the Activity Wheel Counter (Lafayette Instruments Co.). Each wheel revolution was equivalent to 1.1 meters. Wheel revolutions were recorded every 4 hours throughout the entire experimental period.

Microdialysis procedures

On the day of training, CMA 12 high cutoff probes (100 kD cut off; 3 mm; Harvard Apparatus, Holliston, MA) were carefully inserted into cannula sites. The probes were connected to CMA 12 FEP tubing (Harvard Apparatus, Holliston, MA) to deliver aCSF from the CMA 400 syringe pump (Harvard Apparatus, Holliston, MA) to the brain. A sterile 10 μ l micropipette tip (with a capacity of 20 μ l) was inserted into the outlet port of the microdialysis probe via a flexible connector tube (CMA, Harvard Apparatus, Hollister, MA). The dispensing end of the micropipette tip was inserted into the connector tube and the tip opening was covered with parafilm (Pechiney Plastic Packaging, Menasha, WI) to create capillary action to collect and retain the microdialysate in the tip (see Figure 4.1). A minute after probe insertion, the microdialysis procedure began. Throughout the microdialysis procedure, probes were perfused intracranially with aCSF (in mM: 128 NaCl, 2.5 KCl, 1.3 CaCl₂, 2.1 MgCl₂, 0.9 NaH₂PO₄, 2.0 Na₂HPO₄, 1.0 dextrose) at a rate of 0.5 μ l/min while brain dialysate was collected. Each sample was dispensed into microfuge tubes on ice and stored at -20°C until ELISA analyses for BDNF.

One hr was allowed for dialysis to stabilize before any dialysate samples were collected for analysis. Prior to training, dialysate samples were collected from the hippocampus or striatum of each rat for one 90 min baseline measurement while the rat was in its home cage. The dialysate sample taken during training was collected over the following 90 min while rats were trained on the place or response version of the maze. The final sample was a post-training sample collected for 90 min following the cessation of training when the rat was returned to its home cage. After the final sample collection,

rats were overdosed with sodium pentobarbital and brains assessed for verification of probe placement. Rats with misplaced probe insertions or obvious damage that extended beyond the probe placement were excluded from analysis (n=5).

BDNF concentrations in each dialysate sample were analyzed using a Promega Emax BDNF ELISA kit (Promega, Madison, Wisc.) and were compared across training groups and physical activity groups (see detailed methods below).

Behavioral Training

Place and response training were conducted in a 4-arm, plus-shaped maze configured into a “T”. Both tasks required rats to determine the location of a food reward (half of a Frosted Cheerio ©). Rats trained on the place task learned to locate a food reward that was placed in the same region of the room (i.e. east or west arms of the maze; Figure 4.2A). Rats trained on the response task learned to locate a food reward using body movements (i.e. simple right or left turns; Figure 4.2B). Training occurred between 3 and 8 hours after the start of a light cycle.

The maze was constructed of black, 0.64-cm-thick Plexiglas® and the floor of black, matte Plexiglas®. Each of the four arms was 13 cm wide, 18 cm high, and 46 cm long. The center of the maze where all four arms converged was 12 X 12 cm (144 cm²?). The maze was affixed to a 46 cm diameter platform with stops at 90° that allowed for the rotation of the maze. Receptacles with a perforated base were attached to the ends of each maze arm. Inaccessible Frosted Cheerios© were placed beneath each receptacle, so that all arms contained odor cues from the food reward, thereby diminishing the ability of rats to use odor cues to learn the task. In the place training condition, several extramaze visual cues were available to rats. In the response training condition, ceiling-

to-floor sheets surrounded the maze to obstruct access to extramaze visual cues. A lamp with indirect lighting was placed in each corner of the room providing symmetrical ambient light. A fan was used to mask building noise.

Training Procedure

For each rat, start arms were quasi-randomized across twenty trials, so that each start arm (“north” or “south”) was used for 10 trials. For the place task, the goal location (east or west) and for the response task the direction of body turn (right or left) were counterbalanced across rats within a treatment condition.

A trial started when a rat was placed in the start arm and ended after all four paws entered the choice arm. For each trial, the rat was placed in a start arm and given the choice of going down one of the two possible reward arms. For correct, rewarded choices, the rat was allowed to remain in the arm until eating was complete before being returned to its holding cage for the intertribal interval of 30 sec. If the rat chose the incorrect, unrewarded arm, it was allowed to remain for ~5 sec or until it turned to exit before being returned to the holding cage for the intertrial interval. The maximum trial time allowed was 2 min, after which the rat was returned to the cage without making an arm choice. A lack of choice was considered an omission and did not contribute to the number of recorded correct or incorrect choices. Between trials, the maze was rotated 90° to reduce the use of intra-maze cues.

Silver Stain to Determine Total Protein

Silver staining of gels prepared with dialysate samples was performed to determine the molecular weights of the proteins collected by our microdialysis technique. Dialysate samples were combined 2:1 with 2X protein loading buffer (LI-COR

Biosciences, Lincoln, NE) and 10% β -mercaptoethanol and boiled for 10 min prior to being resolved on 4-20% polyacrylamide mini-protean TGX precast gels (BioRad Laboratories, Hercules, CA). Prepared samples (15 μ l) and Kaleidoscope precision plus protein standards (4 μ l; BioRad Laboratories, Hercules, CA) were loaded in triplicate and electrophoresed in SDS-TrisGlycine running buffer at 200 V for 35 min. Following electrophoresis the gels were cut so that each sample and protein standard triplicate were separated from each other. The gel pieces were then set for 20 min in fixative enhancer solution made of 50% methanol (Sigma Aldrich, St. Louis, MO), 10% acetic acid (Sigma Aldrich, St. Louis, MO), 10% fixative enhancer concentrate (BioRad Laboratories, Hercules, CA), in 30% deionized (DI) H₂O. Then gels were rinsed 3 times for 10 min in DI H₂O. Following rinsing, gel pieces were incubated for 7, 15, or 20 min in development solution (BioRad Laboratories, Hercules, CA) prepared according to manufacturer's instructions. The shorter incubation time (7min) allowed us to resolve the molecular weight proteins that were the highest concentration in our samples. However, the shortest incubation time failed to resolve the larger less abundant molecular weight proteins present in our sample. Curious about the molecular weight range of our proteins, we also used series of longer incubation times. Following incubation, the development solution was decanted and a stop solution of 5% acetic acid was incubated on gel sections for 15 minutes. Prior to framing, the gels were rinsed with DI H₂O for 5 min.

Western Blot

Western blot analysis was performed on sample dialysate to detect whether both mature and pro-forms of BDNF were present, because the BDNF ELISA used for BDNF

quantification can detect both pro- and mature forms of BDNF. Dialysate samples were combined 2:1 with (LI-COR Biosciences, Lincoln, NE) 2X protein loading buffer (LI-COR Biosciences, Lincoln, NE) and 10% β -mercaptoethanol and boiled for 10 min prior to running on the gel. Prepared sample dialysate (30 μ l) and Kaleidoscope precision plus protein standards (5 μ l) (BioRad Laboratories, Hercules, CA) were resolved by electrophoresis on a 15% SDS-polyacrylamide gel, run for 75 min at 175 V. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Trans-Blot transfer medium; BioRad Laboratories, Hercules, CA), for 1 hr at 80 V. Following transfer, the nitrocellulose membrane was blocked in 5% non-fat dry milk (SACO Foods Inc., Middleton, WI) and 0.05 M PBS. The nitrocellulose membrane was then incubated at 4 °C for 22 hr in anti-BDNF antibody (Pierce Biotechnology, Rockford, IL) (1:1000) in 1% non-fat dry milk and 0.1% tween. Following primary antibody incubation, the nitrocellulose membrane was washed 4 times for 10 min in 0.05 M PBS and 0.1% tween. Then the nitrocellulose membrane was incubated for 1 hr at room temperature in a secondary antibody of green goat anti-rabbit (1:5000) (LI-COR Biosciences, Lincoln, NE) in 2% non-fat dry milk and 0.1% tween. The membrane was then washed 4 times in 0.05 M PBS and 0.1% tween, rinsed for 2 min in DI H₂O and dried overnight. The following day, the membrane was scanned on the Odyssey infrared detection imaging system (LI-COR Biosciences, Lincoln, NE) to identify the amount and type of protein species present.

BDNF ELISA

Each sample was thawed on ice and diluted (1:6) with 1X block and sample (B&S) buffer (Promega, Madison, WI). Sandwich-style enzyme-linked immunosorbent

assays (ELISAs) were performed to detect BDNF using the BDNF Emax ImmunoAssay System kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, the 96-well plate was coated with anti-BDNF mAb and refrigerated overnight (16-20 hr). The following day, the wells were blocked for non-specific binding with 1X B&S buffer for 1 hr. Samples and standards (ranging from 30, 15, 7.5, 3.75, 1.87, 0.93, and 0 pg/ml) were added (100 µl/well) to the coated wells and incubated on a plate shaker for 2 hr at room temperature. The wells were then washed (5 X) with 1X B&S buffer and incubated and shaken with 100 µl anti-human BDNF pAb for 2 hr. Following another wash (5X), the wells were incubated and shaken with Anti-IgY HRP conjugate for 1 hr. Following a final wash, 100 µl of HRP substrate (TMB One solution) was added to the wells, which were shaken for 10 min. The oxidation reaction was stopped with 100 µl of 1N HCl. The absorbance of each well was read at a wavelength of 450 nm (within 10 min of stopping the reaction) in a Fischer Scientific ThermoMax microplate reader with Ascent software (Fischer Scientific, Pittsburgh, PA). BDNF content was interpolated from standard curves run on each plate. In previous Chapters 2 and 3, we measured only mBDNF with this same ELISA kit. However, in the current study both mBDNF and proBDNF were detected in the microdialysis samples. The difference between the current study and previous studies (Chapters 2 and 3) is that we are using extracellular fluid instead of brain homogenate. When using brain homogenate it is necessary to acid treat the samples to measure both pro and mature forms of BDNF. However, since we are using extracellular fluid in the current study, no acid treatment is necessary to recover both forms of BDNF. Thus, this particular ELISA protocol is measuring both pro and mature forms of BDNF.

Statistical analysis

Mean baseline ECF BDNF levels in the hippocampus and striatum were compared between place- and response- trained sedentary rats using unpaired, two-tailed Student's *t*-tests. Changes in ECF BDNF in the hippocampus and striatum during and after place and response learning were determined by calculating difference scores from baseline levels and used as the dependent measure for comparisons between activity groups, between learning tasks, and their interaction. Repeated measures analysis of variance (ANOVAs) were used to determine changes in ECF BDNF within brain region. Two-way ANOVAs were used to measure task x brain region interactions in the hippocampus and striatum of place and response trained sedentary rats. Paired Student's *t*-tests were performed to compare post training concentrations from baseline concentrations of ECF BDNF in the hippocampus and striatum following place and response training. To compare % correct across implant sites (hippocampus and striatum) and task (place and response) a 2-way ANOVA was conducted. To compare the baseline ECF BDNF concentrations in exercise and sedentary rats in the hippocampus and striatum a 2-way ANOVA was performed. One-way repeated measures ANOVAs were used to determine changes in ECF BDNF across place and response training. Repeated measures ANOVAs were used to determine differences in ECF BDNF between brain regions in exercise rats during training. One-way repeated measures ANOVAs were used to determine changes in raw ECF BDNF in the striatum of exercise and sedentary rats. Repeated measures ANOVAs were used to measure differences across the average daily distance run per week in rats that had hippocampus and striatum implants. Statistical significance was set at $\alpha = 0.05$.

All procedures followed the institutional and federal guidelines and were approved by the IACUC at the University of Illinois.

RESULTS

Examination of the silver stained gels of our samples showed that the dialysate contained proteins with molecular weights that ranged from 10-75 kD. There appeared a rather dramatic cut-off at ~75kD. The three exposure times for the silver staining allowed visualization of the range of molecular weights of proteins in our sample. The shorter incubation time allowed detection of proteins that were the highest concentrations in our sample, while the longest incubation times showed the full range of protein molecular weights in our sample. For example, after 7 min of silver stain exposure there were protein species at ~14 kD and ~70 kD (Figure 4.3A). After 15 min, there were several protein species spanning from 10 kD to 75 kD (Figure 4.3B), and after 20 min the smear of proteins ranged from 10 kD to 75 kD but did not exceed 75 kD (Figure 4.3C). At all exposure times, the 14 kD and 70kD bands were dramatically larger than were bands at other molecular weights (Figure 4.3A-C).

The western blot detected bands, ranging between 10-15 kD when incubated with anti-BDNF antibody. Protein bands at these molecular weights correspond with mature (Figure 4.3D).

There were no differences in baseline values of ECF BDNF between rats assigned to place training and rats assigned to response training for both the hippocampus ($t_{11}=0.03$, $p > 0.2$) and striatum ($t_{11}=0.58$, $p>0.2$; data now shown). Interestingly, baseline values of ECF BDNF were significantly different between the hippocampus and striatum ($t_{24}=3.31$, $p<0.01$) with concentrations from the hippocampus

(2.8 pg/ml \pm 0.57 pg/ml) more than three times concentrations found in the striatum (0.84 pg/ml \pm 0.18; Figure 4.4).

Responses of ECF BDNF to training on the two different tasks were compared within structure to determine whether cognitive strategy required by the task altered the pattern of BDNF release. In the hippocampus, ECF BDNF concentrations increased during place learning but decreased during response learning (Figure 4.5A). The opposite was seen in the striatum, where ECF BDNF levels increased during response learning and decreased during place learning (Figure 4.5B). This pattern of results can be seen as a response to training measured as a change from baseline. There was no main effect of either task ($F_{(1,22)}=1.3$, $p>0.2$) or brain region ($F_{(1,22)}=0.52$, $p>0.2$) on the change in ECF BDNF in the hippocampus (Figure 4.5A) and striatum (Figure 4.5B) during place and response training. However, there was a significant interaction effect of task and brain region ($F_{(1,22)}=14$, $p<0.001$), highlighting the double dissociation in BDNF response between task and brain structure. When analyzed within brain structure, hippocampal ECF BDNF was significantly different in place and response trained rats ($t_{11}=2.7$, $p<0.05$; Figure 4.5A) while striatal ECF BDNF was not ($t_{11}=2.06$, $p=0.06$; Figure 4.5B).

After training when rats were removed from the maze, the ECF BDNF in the hippocampus returned to baseline, as values were not different from baseline following place ($t_{12}=0.19$; $p>0.2$) or response training ($t_{10}=1.5$; $p<0.2$). The post-training ECF BDNF levels in the hippocampus were also not different from training following place ($t_{12}=0.3$; $p>0.2$) or response ($t_{10}=0.19$; $p>0.2$) training. In the striatum, the ECF BDNF concentrations returned to baseline after place training ($t_6=0.2$; $p>0.2$), but continued to

increase following response training. However, ECF BDNF values at the post-training time point were not statistically different from baseline values ($t_{16}=1.8$; $p=0.07$) and were not different from response training values ($t_{16}=0.6$; $p>0.2$).

For percent correct on the place and response tasks there was a main effect of task ($F_{(1,21)}=15$, $p<0.001$) and brain region ($F_{(1,21)}=9.6$, $p<0.01$), however there was no interaction between task and brain region ($F_{(1,21)}=0.014$, $p>0.2$). Regardless of the implant site (hippocampus or striatum), rats performed better on the response task than on the place task, as seen by higher percent correct choices across the whole training period (hippocampus: $t_{11}=2.6$, $p<0.05$; striatum: $t_9=2.94$, $p<0.05$; Figure 4.6). Moreover, there were significant differences in the percent correct on the place task ($t_9=2.68$, $p<0.05$) across rats with intrahippocampal and intrastriatal probes. There were not statistically significant differences in learning the response task across rats with intrahippocampal and intrastriatal probes ($t_{12}=2.02$, $p<0.1$).

Physical activity modulated ECF BDNF in the hippocampus and striatum in different ways. There was no main effect of brain region ($F_{(1,29)}=0.82$, $p>0.2$) or exercise condition ($F_{(1,29)}=2.02$, $p<0.2$) on baseline ECF BDNF levels. There was no effect of an interaction between brain region and exercise condition ($F_{(1,29)}=1.82$, $p<0.2$) on baseline ECF BDNF levels. The baseline ECF BDNF concentrations in the hippocampus were the same in sedentary and exercise rats ($t_{15}=0.08$, $p>0.2$; Figure 4.7A). In the striatum, ECF BDNF was elevated before response training in Exercise rats compared to sedentary rats, although the increase did not reach statistical significance ($t_{14}=1.92$, $p<0.1$; Figure 4.7B). Striatal baseline concentrations of ECF BDNF in exercise rats actually rose to the levels measured in the hippocampus of sedentary and exercise rats.

Physical activity had different effects on the hippocampal and striatal responses of ECF BDNF to training in the canonical tasks. For example, using change from baseline, priming with exercise produced significantly elevated ECF BDNF responses to place learning in the hippocampus ($F_{(2,18)}=5.34$, $p<0.05$; Figure 4.8A). In contrast, exercise failed to potentiate the increase in striatal ECF BDNF during response learning ($F_{(2,12)}=0.8$, $p>0.2$; Figure 4.8B) and compared to the response measured in sedentary controls. This interaction between exercise status and brain structure/task on the ECF BDNF levels was seen as a trend that did not quite reach significance ($F_{(2,30)}=2.8$, $p<0.1$). Because mean baseline values of ECF BDNF in the striatum tended to be elevated in exercise rats, evaluation of raw BDNF values across the training period revealed that BDNF concentrations in the striatum of exercise rats were consistently, though not significantly, higher than levels in the sedentary controls ($F_{(1,14)}=3.6$, $p<0.1$; Figure 4.9). Viewed in this way, striatal ECF BDNF in exercise rats was high prior to training and remained high through training whereas levels in sedentary rats rose during training and remained high after training (Figure 9).

Distances run and accuracy levels during training were the same in rats with hippocampus and striatum implants. Rats with hippocampus and striatum implants demonstrated substantial running that increased across the three-week period (hippocampus: $F_{(2,18)}=10$, $p<0.05$; Figure 4.10A; striatum: $F_{(2,12)}=4.9$, $p<0.05$; Figure 4.10B). For example, daily running accumulated more than 20 m during week one that rose to over 100 m during week three. During week three when food restriction was conducted, there was elevated running. There were no differences in distance run between rats with hippocampus or striatum implants across any time point ($F_{(2,30)}=0.03$,

$p > 0.2$). Three weeks of access to running wheels failed to enhance learning in place ($t_{13} = 0.24$, $p > 0.2$) or response ($t_{15} = 0.4$, $p > 0.2$) tasks (data not shown).

There was a slight increase in ECF BDNF in the hippocampus and a slight decrease in ECF BDNF in the striatum during SA testing, but these changes in ECF BDNF from baseline were not significant. During SA testing there was no change from baseline in ECF BDNF levels in the hippocampus ($t_4 = 0.8$; $p > 0.2$; Figure 4.11A) or striatum ($t_4 = 2.28$; $p < 0.1$; Figure 4.11B). In rats that were cognitively primed with SA testing, there was no change in ECF BDNF in the hippocampus during place training ($t_4 = 1$; $p > 0.2$; Figure 4.11A) and an increase in ECF BDNF in the striatum during response training, but this increase in striatal ECF BDNF was not statistically significant ($t_4 = 1.4$; $p > 0.2$; Figure 4.11B). Place and response learning scores were above 50% correct in the response task, but at 50% correct (chance) in the place task (Figure 4.10C). SA testing scores were above chance (44%) (Figure 4.11D).

DISCUSSION

This report marks the first published study of *in vivo* measurements of extracellular BDNF in rats moving on a T-maze and engaging in hippocampus- and striatum-sensitive learning strategies. The use of the microdialysis method provided the first evidence of BDNF release being modulated by learning strategies in the hippocampus and striatum. Additionally, the microdialysis method was capable of providing evidence of the differences in learning-induced BDNF release in the hippocampus and striatum following voluntary exercise.

The microdialysis technique has been particularly well validated for the *in vivo* monitoring of extracellular levels of neurotransmitters, and less so for peptides,

hormones, and neurotrophic factors. Although there are several studies, including reports from our lab, that use the microdialysis method in the brains of awake behaving rats (Chang and Gold, 2003; Pych et al., 2005a; 2005b; Newman et al., in prep; Ragozzino et al., 1998), none have yet measured BDNF (for review see Shippenberg and Thompson, 2001; Humpel et al., 1995). In fact, microdialysis methods have been successfully used to measure extracellular neurotrophic factors in the hippocampus and striatum of awake rats prior to and following administration of kainic acid (Humpel et al., 1995). However, in this report the bioassays and enzyme immunoassays (EIA) used to quantify neurotrophic factor release were specific to the neurotrophic factor NT-3 (Humpel et al., 1995). Moreover, the authors provided confirmation that the neurotrophic factor NT-3, not BDNF, increased in the hippocampus following the administration of the excitatory drug kainic acid. Although the microdialysis method did successfully collect neurotrophic factors of the same molecular weight as BDNF, evidence of extracellular BDNF in the striatum or hippocampus was not reported (Humpel et al., 1995). Furthermore, Humpel et al., 1996 argue that neurotrophic factors are difficult to capture in conditions when the animal is moving. Thus, their experiments never measured neurotrophic factors in rats engaged in cognitive tasks.

This is the first study to measure release of BDNF in the contexts of cognitive and physical activity. BDNF measurements from brain homogenate are not on their own useful for understanding the role of activity-dependent BDNF release in learning because BDNF protein content in whole tissue homogenate is a combination of several non activity-dependent forms of BDNF (i.e. vesicular, receptor bound, intracellular trafficking forms, and constitutively released forms of BDNF). In addition, the

microdialysis technique allows for simultaneous drug infusion during dialysis (Qi and Gold, 2009; Sadowski et al., 2011). It is possible to use this technique to gain more information concerning cognitive and physical activity-induced BDNF release in the presence of pharmacological agents in the brain. For example, given that BDNF-TrkB signaling in itself is hypothesized to play a role in potentiating BDNF release (Bramham and Messaoudi et al., 2005; Canossa et al., 1997), this technique could be used to measure BDNF release in the presence of TrkB inhibitors, in physically active and sedentary rats.

Our silver stains demonstrated that our samples contained protein sizes ranging from 10-75 kD. Thus, our microdialysis method is likely collecting both pro (32 kD) and mature (14 kD) forms of BDNF. Western blots of our sample dialysate further confirmed the presence of mature BDNF species. Given that the BDNF ELISA we used is sensitive to both pro and mature forms of BDNF, the BDNF concentrations reported here include both pro and mature BDNF.

Both pro and mature BDNF are released in an activity-dependent manner (Yang et al., 2009; Mowla et al., 2001; for review see Cunha et al., 2010). Upon release, proBDNF is proteolytically converted to mBDNF by extracellular proteases (Seidah et al., 1996; for review see Greenberg et al., 2009; Lu, 2003; Lu, 2005). The extracellular conversion of pro to mature BDNF appears to be important for learning and memory processes; and inhibition of the extracellular proteases (i.e. tissue plasminogen activator (tPA)) interferes with synaptic plasticity and learning and memory processes (Pang et al., 2004; Barnes and Thompson 2008). Furthermore, the impairments induced by tPA inhibition is reversed with mBDNF administration (Pang et al., 2004). In fact, tPA

increases in an activity-dependent manner. For example, membrane depolarization and physical fitness increases tPA activity (Gualandris et al., 1996; Baranes et al., 1998; Ding et al., 2011). Thus, using the microdialysis method in contexts of cognitive and physical activity to quantify extracellular mBDNF and proBDNF separately will be an important future direction of these findings.

The use of the microdialysis technique demonstrated that the hippocampus and striatum have different concentrations of ECF BDNF at baseline. Others have reported that the hippocampus and striatum release BDNF via different mechanisms. For example, in the hippocampus, BDNF is released both pre- and post-synaptically (Hartmann et al., 2001; Edelmann et al., 2014). However, in the striatum BDNF is released only presynaptically (Jia et al., 2010). Given this evidence, it is plausible that the hippocampus has higher levels of ECF BDNF compared to the striatum. For example, ECF NT3 levels are reduced in the striatum compared to the hippocampus (Humpel et al., 1995). In addition, others have shown the hippocampus has higher levels of BDNF mRNA and protein compared to the striatum (Conner et al., 1997; Croll et al., 1998).

The differences in extracellular BDNF concentrations in the hippocampus and striatum during place and response training demonstrated the sensitivity of BDNF release to learning strategies. For example, when the hippocampus is actively engaged during the place task or the striatum during the response task, BDNF release increases in the canonical structure to support learning. It is possible that noncanonical areas (i.e. the hippocampus during the response task and the striatum during the place task) inhibit BDNF release during task learning to actively eliminate competition between

memory systems. Previously we have shown that inhibition of BDNF-TrkB signaling in the hippocampus and striatum attenuates priming-induced enhancements in place and response learning by either previous cognitive or physical activity (Chapters 2 and 3; Korol et al., 2013). Thus, it is possible that if we inhibited BDNF-TrkB signaling in the noncanonical structure prior to training, we would see enhancements in place and response learning.

Previously, our lab has shown that neurochemical shifts occur in the hippocampus and striatum, reflecting the cognitive strategies engaged. For example, extracellular acetylcholine, glucose, and lactate increase in the hippocampus during place learning and the striatum during response learning (Chang and Gold, 2003; Gold et al., 2013; Pych et al., 2005a and b; Newman et al., in prep). Furthermore, the hippocampus and striatum have no change or decrease in extracellular acetylcholine when rats are engaged in a noncanonical task (i.e. response learning for the hippocampus and place learning for the striatum) (Chang and Gold, 2003; Gold et al., 2013). In the current study, increases in BDNF release were also brain region- and task-specific. Taken together, these data provide further neurochemical evidence for the hippocampus and striatum as interacting neural systems during learning and memory.

The specificity of increased BDNF release due to learning was further demonstrated by analyzing post-training ECF BDNF samples. In the hippocampus, ECF BDNF levels returned to baseline following either place training, and slightly decrease from baseline following response training. Interestingly, in the striatum ECF BDNF levels returned to baseline following place training, but continued to increase following response training. Taken together, these results suggest that ECF BDNF levels are

sensitive to learning events in both the hippocampus and striatum, and that BDNF release in the striatum increases during the learning of a canonical task but not during the learning of a non-canonical task. Previously we have shown that whole tissue BDNF content in the striatum, but not the hippocampus, increases 24 hr following a working memory task (Scavuzzo et al., in prep). It is possible that the long-lasting increase in striatal, but not hippocampal, BDNF content following spontaneous alternation testing is due to increased BDNF release in the striatum, but not the hippocampus, following the task. In a rewarded spontaneous alternation task, the striatum increases acetylcholine release compared to the hippocampus in the later stages of SA testing (Pych et al., 2005). Although the rewarded version of spontaneous alternation testing likely leads to a shift in striatal strategy, it is possible that the striatum engages in spontaneous alternation testing later in the testing period, potentially leading to increases in BDNF following testing.

The current findings demonstrate that physically active, but not sedentary rats had significantly increased ECF BDNF from baseline in the hippocampus during place learning. ECF BDNF in the striatum of physically active rats tended to be increased prior to, during, and following response learning compared to sedentary rats. Voluntary exercise has previously been shown to increase tissue levels of BDNF protein in the hippocampus and striatum (Ding et al., 2003; Berchtold et al., 2005; Korol et al., 2013; Scavuzzo et al., in prep). In addition, several studies have found that exercise treatments enhance hippocampal- and striatal-sensitive learning (Fordyce and Farrar, 1991; Anderson et al., 2000; Van de Bught et al., 2007; Korol et al., 2013) and that BDNF signaling may underlie exercise-induced learning enhancements (Vaynman et

al., 2004; Korol et al., 2013). Although informative, these studies have been unable to elucidate the mechanism by which increases in BDNF protein and signaling play a role in exercise-induced learning enhancements. Taken together with previous findings (Korol et al., 2013), the findings in the current study—increased ECF BDNF in the striatum prior to and during response learning, and in the hippocampus during place learning—provide further knowledge and understanding of the mechanism by which exercise enhances place and response learning. The data in the current and past studies (Korol et al., 2013; Adlard et al., 2004; Ding et al., 2003; Berchtold et al., 2005) suggest that physically active rats have increased storage and release of BDNF protein in the striatum and increased release of BDNF in the hippocampus during task learning compared to sedentary rats. Whether or not the learning-induced potentiation in ECF BDNF in the hippocampus of exercise rats results from alterations in BDNF metabolism is not yet known.

In the current study, the effects of voluntary exercise enhancing place and response learning performance were not replicated. Of note, the rats in the current study ran much shorter distances over the 3-week running period compared to previous studies (Scavuzzo et al., in prep). It is possible that the decreased running distances in the current study contributed to the lack of exercise effects on learning. Given that the microdialysis protocol pulls BDNF, other large proteins, neurotransmitters, and ions (for review see Shippenberg and Thompson, 2001) out of the ECF, it is possible that the microdialysis protocol interferes with the mechanisms that support exercise-induced learning enhancements. Past studies in our lab using microdialysis to identify mechanisms of enhancements in learning due to estrogen treatment have also found a

lack of an effect of estrogen on place learning (Marriott and Korol, 2003). In the future to test if microdialysis is affecting learning in exercise rats we could turn off ECF collection during training to see if this affects learning scores.

Cognitive priming with SA had no effect on ECF BDNF levels in the hippocampus and striatum during place and response training. Given that the experimental procedure to test for SA-induced changes in ECF BDNF during place and response training is different (microdialysis occurring for 90 min longer before place or response training, changing training rooms between SA testing and place or response training, and shutting the microdialysis pump on and off to switch rooms) it is likely that the different methodologies altered our ability to replicate our previous findings –increased ECF BDNF in the hippocampus during place learning and in the striatum during response learning. We wanted to test the hypothesis that priming with cognitive and physical activities enhance place and response learning via similar signaling mechanisms (see Chapters 1-3). In the current study, we found that physical activity increased ECF BDNF in the hippocampus and striatum during place and response learning, respectively. However, we were unable to show that cognitive priming with SA has any effect on ECF BDNF in the hippocampus or striatum before, or during place or response learning, respectively. Currently, our lab is attempting to design and execute studies that can overcome the methodological differences seen in the current study, so as to properly test the hypothesis that cognitive and physical activity are enhancing place and response learning via similar changes in BDNF release in the hippocampus and striatum.

The current study provides a novel, relatively simple method of measuring ECF BDNF in contexts of cognitive and physical activity. The data presented here provide the first published evidence of BDNF release in the hippocampus and striatum in response to engagement in canonical tasks. Our findings also highlight the differences in the patterns of BDNF release in the hippocampus and striatum. For example, after training on either task hippocampal ECF BDNF fails to rise whereas striatal BDNF continues to increase. In addition, this study provides further insight into the mechanisms underlying exercise-induced learning enhancements. We also report differences in the patterns of BDNF release in the hippocampus and striatum in physically fit animals in response to canonical task training. Our data suggest that exercise-induced enhancements in response learning may be mediated by increases in BDNF release prior to training, while exercise-induced enhancements in place training may be mediated by increases in BDNF release during training. Thus, the use of the microdialysis method to measure ECF BDNF can provide a valuable understanding of the mechanisms of BDNF release in the context of cognitive and physical awake behaving animals.

The microdialysis method used in this study can aid in understanding the time frame of BDNF release and signaling in response to physical fitness and learning strategies. In addition, the use of the BDNF microdialysis technique could aid in elucidating the role of BDNF release in development, plasticity, aging, depression, and neurodegeneration. Decreases in BDNF protein and signaling have been implicated in the development of diseases such as Alzheimer's and depression (Lee et al., 2005; Martinowich et al., 2007; Phillips et al., 1991; Zuccato and Cattaneo 2009) but it is

unknown if the mechanisms of BDNF release are altered in these brains. Additionally, it would be interesting to measure whether drugs used to treat these diseases were capable of changing BDNF release. Thus, the method of measuring *in vivo* ECF BDNF levels will give us a better understanding of the role of BDNF signaling and release across models of development, disease, and pharmacological treatments.

FIGURES

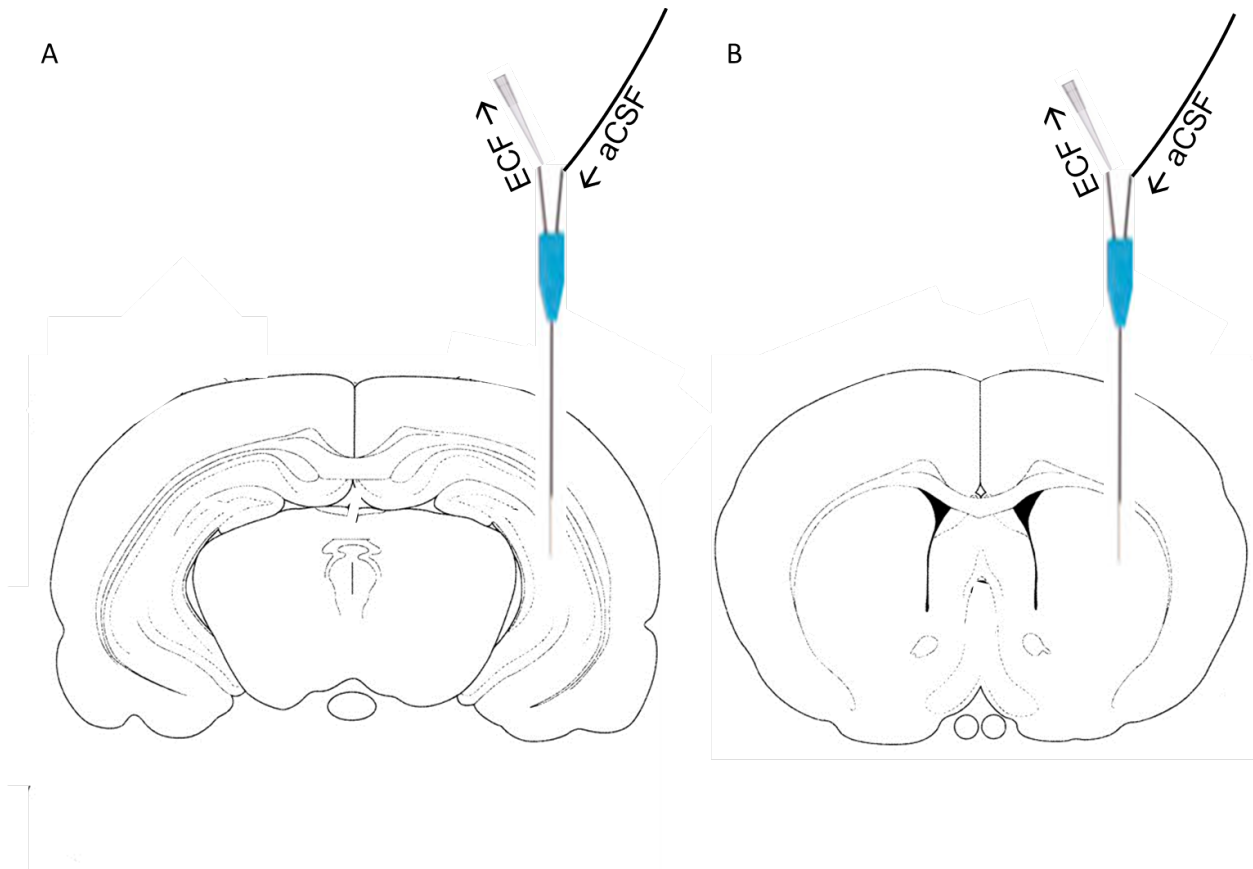
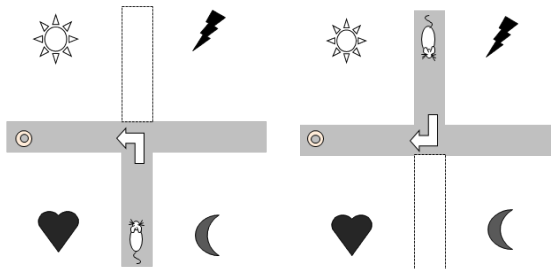


Figure 4.1. Diagram of hippocampus and striatum microdialysis probe implants.

Cannulae were implanted above the ventral hippocampus (A) or lateral striatum (B). On the day of training microdialysis probes were inserted via the cannula with a 3 mm projection into the brain area for collection of extracellular fluid. Tubing was connected to the microdialysis probe for perfusion into and collection from the region of interest. aCSF was perfused into the brain and ECF was collected into a micropipette tip.

A

Place Learning



B

Response Learning

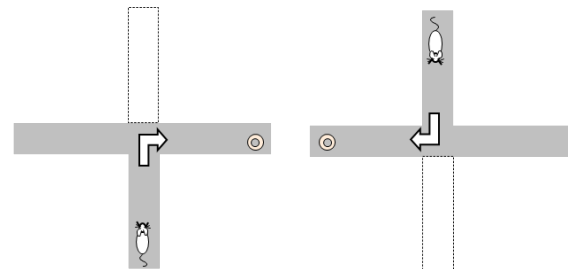


Figure 4.2. Place and response training. In T-shaped mazes rats were trained on either the A) place or B) response tasks. In A) place training, rats solved the maze by retrieving a food reward in the same location in the room, using extramaze cues. In B) response training, rats solved the maze by retrieving a food reward using the same egocentric body movement (i.e. left or right turns).

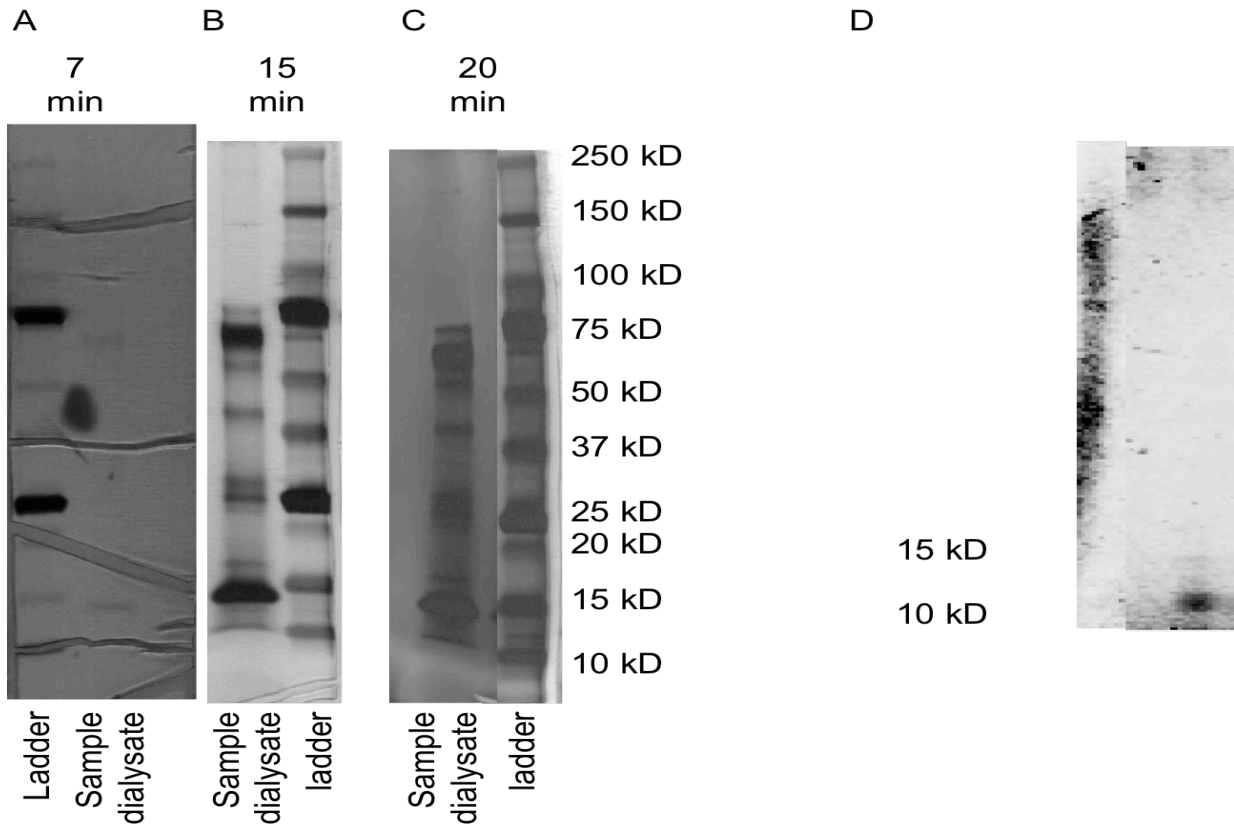


Figure 4.3. Silver stain and Western blot of brain ECF samples. Silver stains of the sample dialysate demonstrate that proteins in the ECF collected ranged from ~10 kD to ~75 kD. In A) the 7 min silver stain incubation only proteins of molecular weights of 14 kD and 70 kD were present. In B) the 15 min silver stain incubation proteins of molecular weights ranging from 10 kD to 70 kD were present. In C) the 20 min silver stain incubation proteins of molecular weights ranging from 10 kD to 70 kD were present. D) A Western blot using anti-BDNF primary antibody detected protein bands at between 10-14 kD, indicating the presence of mature BDNF in the ECF collected in the sample dialysate.

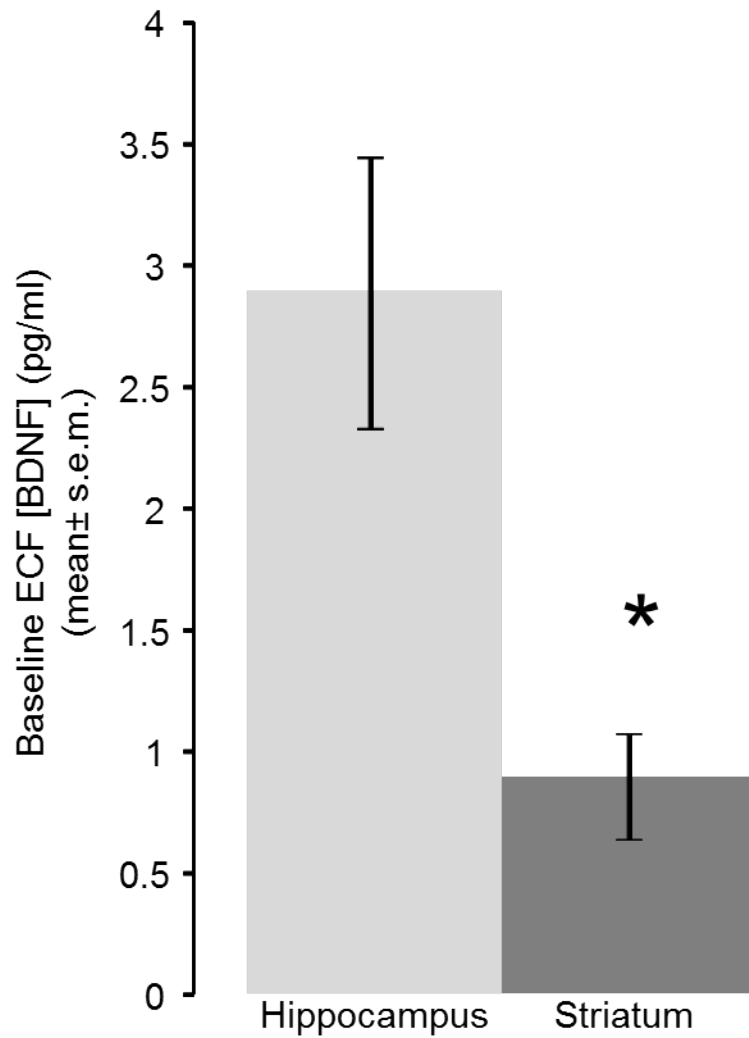


Figure 4.4. Baseline ECF BDNF measurements in the hippocampus and striatum.

Prior to learning either task, ECF BDNF levels are significantly higher in the hippocampus compared to the striatum in sedentary rats. $*=p<0.05$. $N=13$ for hippocampus, $N=13$ for striatum.

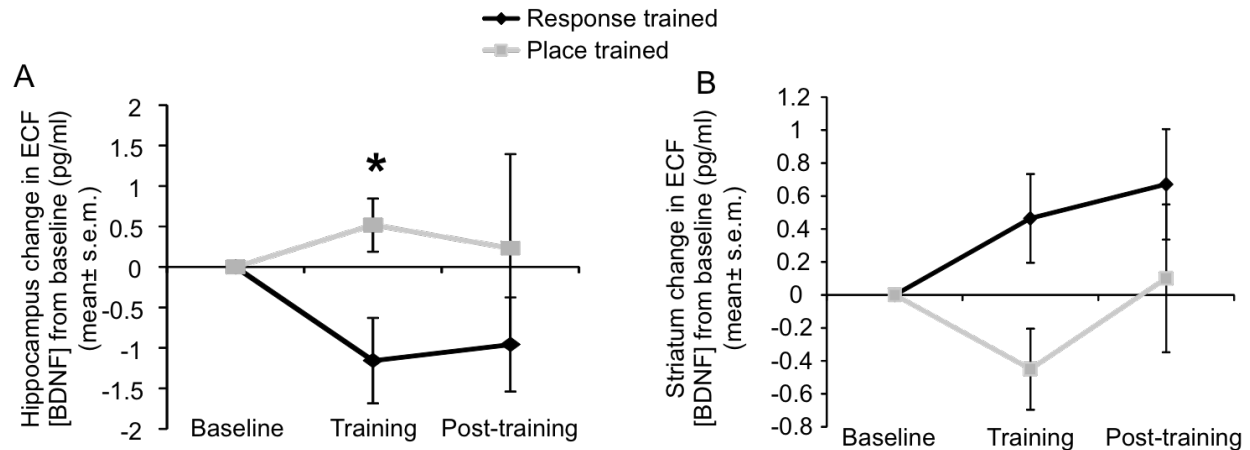


Figure 4.5. Change in hippocampus and striatum ECF BDNF during place and response learning. A) The change in hippocampal ECF BDNF levels from baseline was significantly different during place vs response training. Neither task elicited significant changes from baseline. B) The change in striatal ECF BDNF levels from baseline was increased during response training and decreased during place training. During the post-training period, ECF BDNF returned to baseline in the place-trained rats, whereas ECF BDNF tended to remain elevated in response-trained rats, but this increase from baseline was not significant. $*=p<0.05$ place vs. response training; striatum place vs response training $p=0.063$. $N=7$ for hippocampus place training, $N=6$ for hippocampus response training, $N=4$ for striatum place training, $N=9$ for striatum response training.

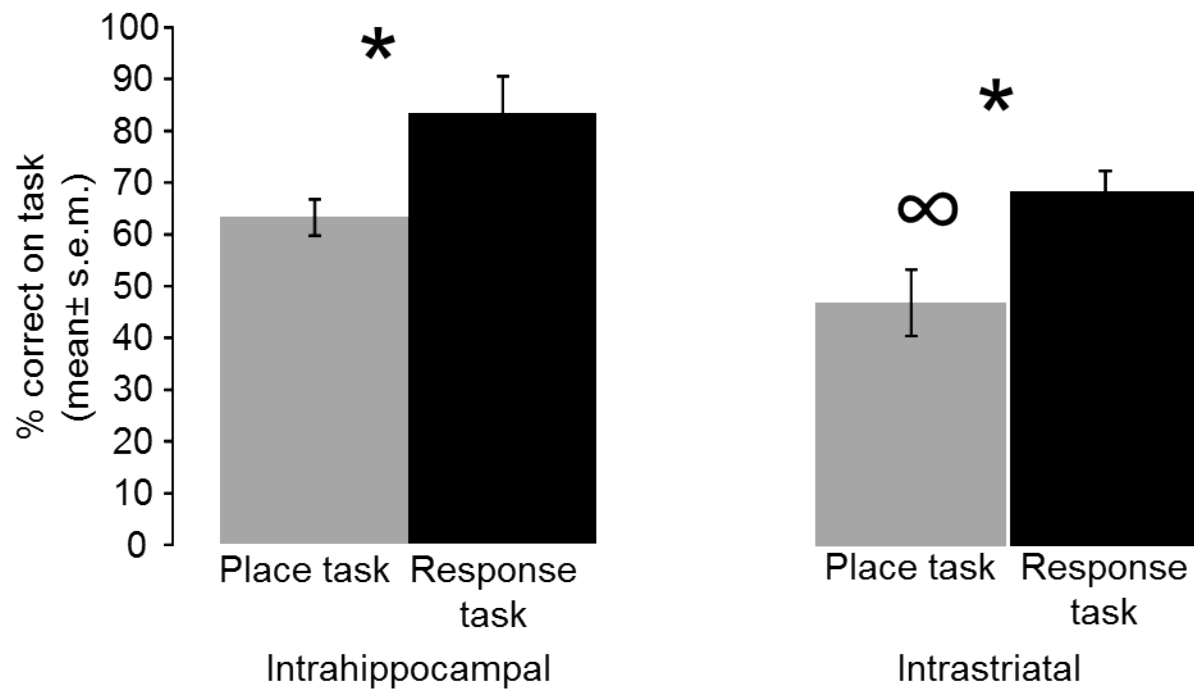


Figure 4.6. Place and response learning scores in rats with hippocampal and

striatal implants. Rats that were response trained had higher % correct scores

compared to rats that were place trained in rats that had hippocampus implants and in

rats that had striatum implants. Rats that had hippocampal implants had higher %

correct scores on the place task compared to rats that had striatal implants. $*=p<0.05$

place vs. response; $\infty=p<0.05$ vs. place intrahippocampal. $N=7$ for intrahippocampal

place training, $N=6$ for intrahippocampal response training, $N=4$ for intrastratial place

training, $N=9$ for intrastratial response training.

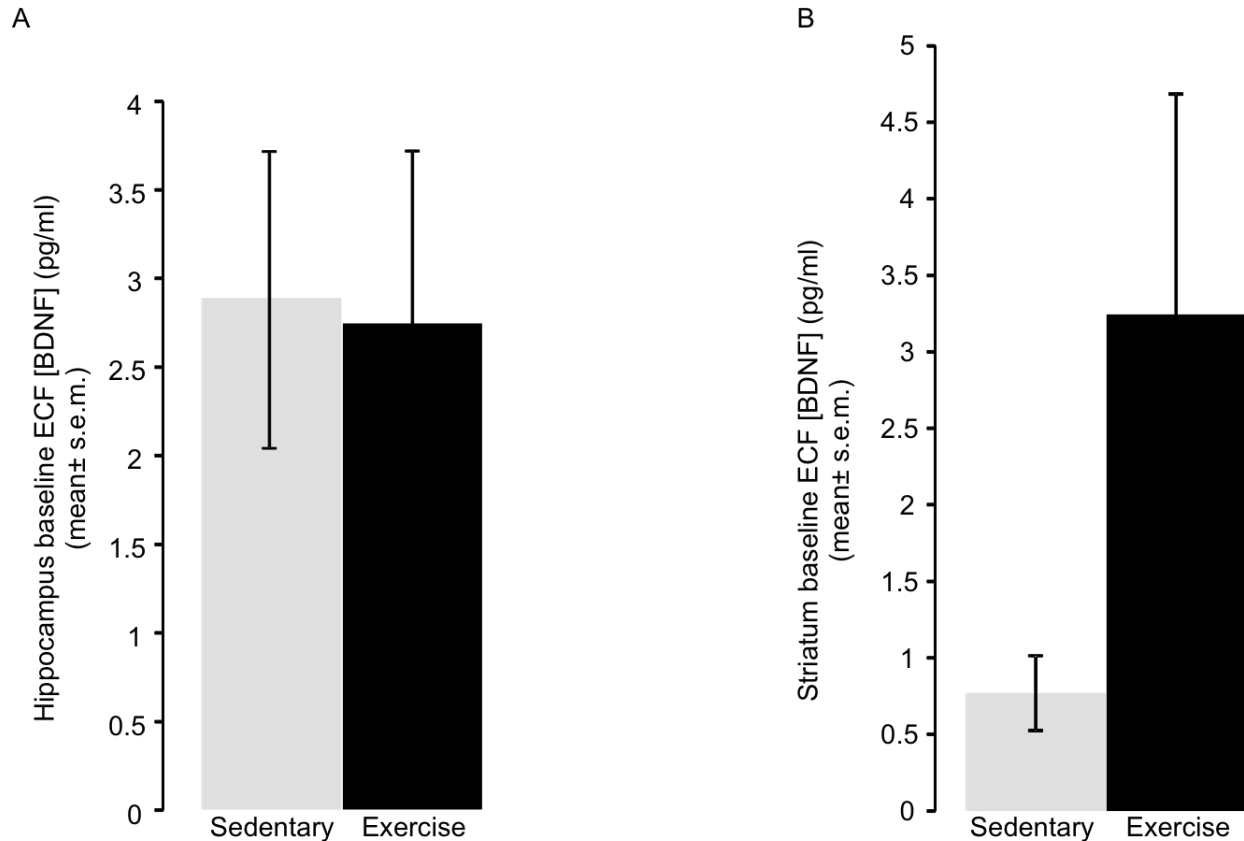


Figure 4.7. ECF BDNF in the hippocampus and striatum of sedentary and exercise rats. In A) there is no difference in the baseline ECF BDNF levels in the hippocampus of exercise and sedentary rats. In B) the ECF BDNF levels in the striatum of exercise rats are higher than sedentary rats; however this increase is not statistically significant. Striatum exercise vs. sedentary, $p=0.075$. For hippocampus $N=7$ for sedentary and $N=10$ for exercise; for striatum $N=9$ for sedentary and $N=7$ for exercise.

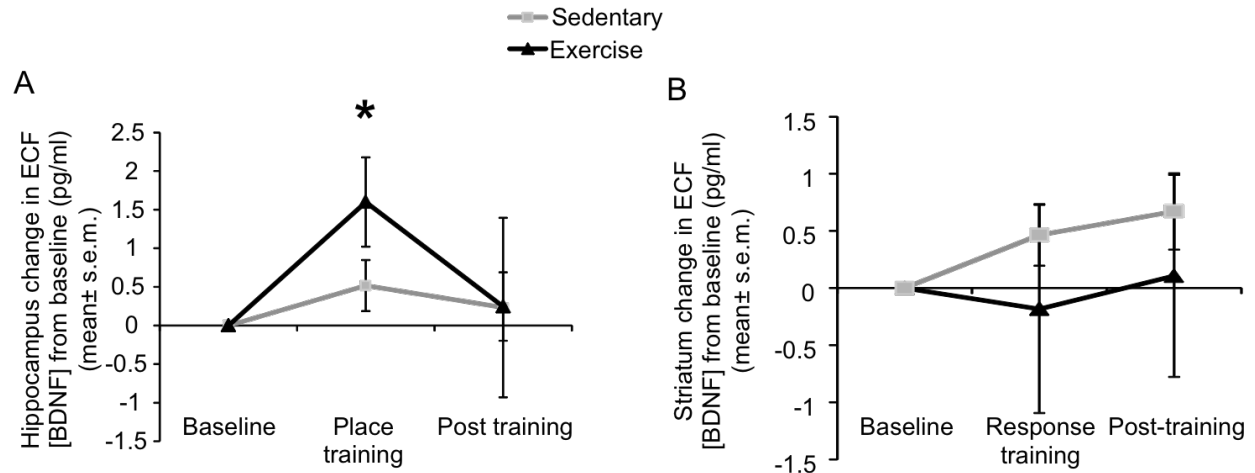


Figure 4.8. ECF BDNF in physically active rats before, during, and following response training. In A) ECF BDNF in the hippocampus increased significantly from baseline during place training in physically active, but not sedentary rats. In B) ECF BDNF in the striatum did not significantly change from baseline in physically active rats during or following response training. *= $p < 0.05$. For hippocampus $N=7$ for sedentary and $N=10$ for exercise; for striatum $N=9$ for sedentary and $N=7$ for exercise.

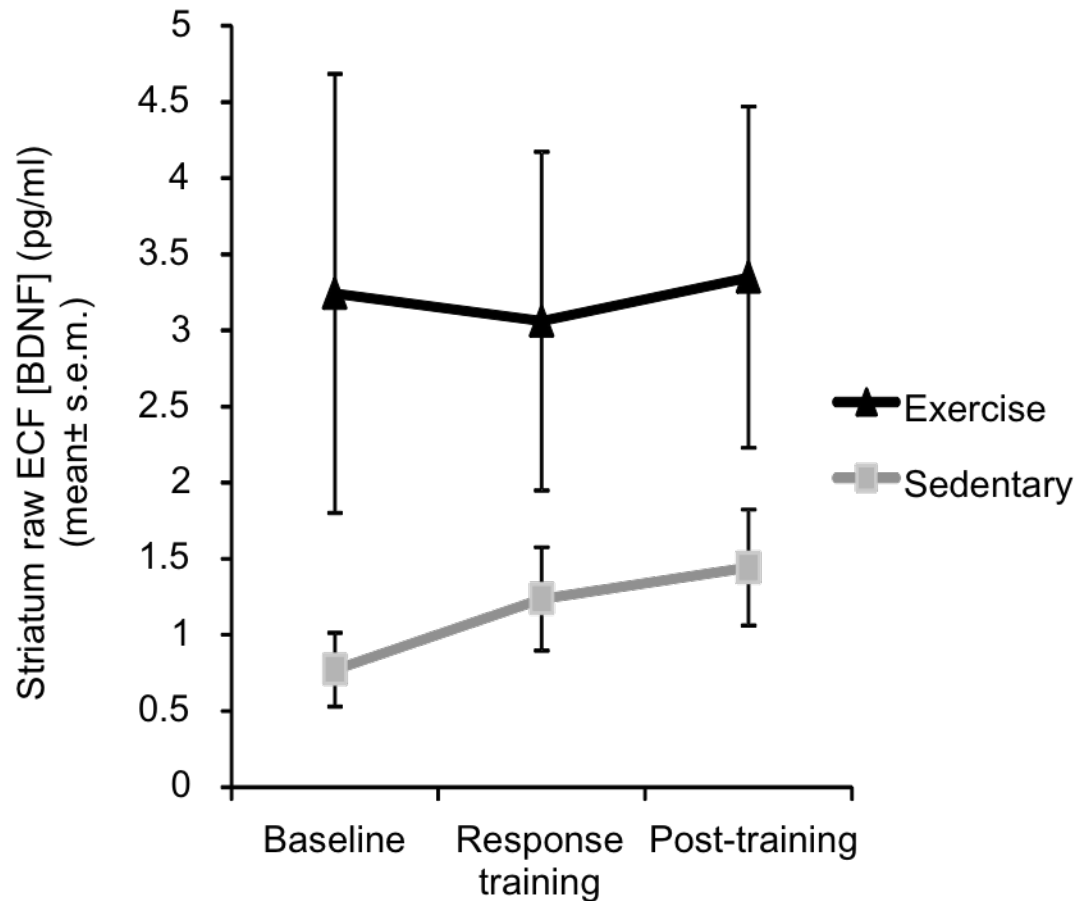


Figure 4.9. Raw ECF BDNF in the striatum of sedentary and exercise rats. Raw levels of ECF BDNF were increased in the striatum before, during, and following response training in physically active compared to sedentary rats, however this effect was not statistically significant. Interestingly, although sedentary rats increased ECF BDNF levels during and following response training, striatum ECF BDNF levels in sedentary rats never did increase to the levels observed in physically active rats. Exercise vs. sedentary $p=0.091$. $N=9$ for sedentary, $N=7$ for exercise.

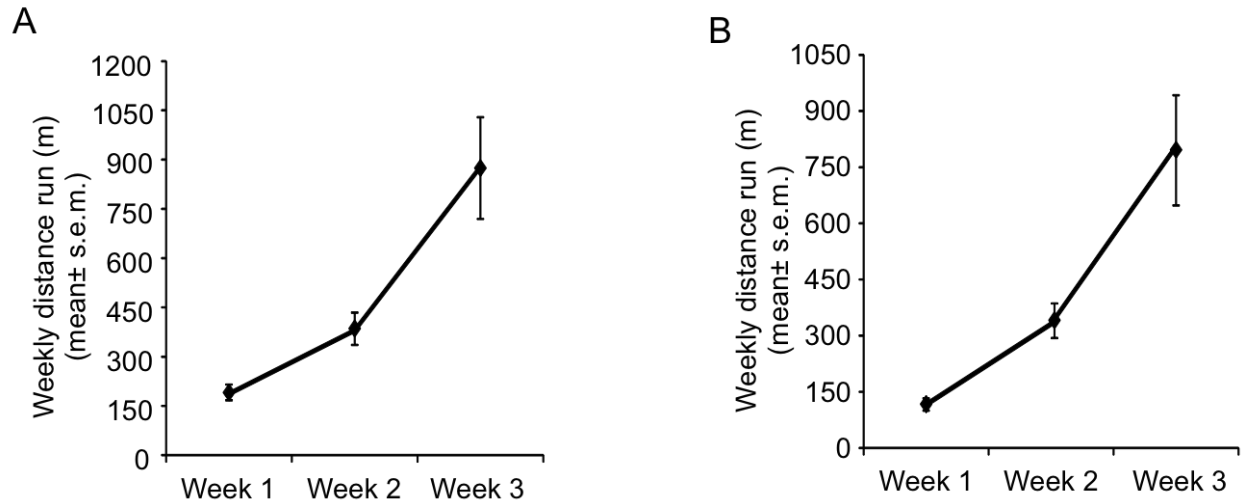


Figure 4.10. Average daily distance run per week in hippocampus and striatum

implanted rats. In A) hippocampus-implanted and B) striatum-implanted rats all rats with access to running wheel ran during the 3-week running period and increased their running distance every week, especially in the third week, when undergoing food restriction.

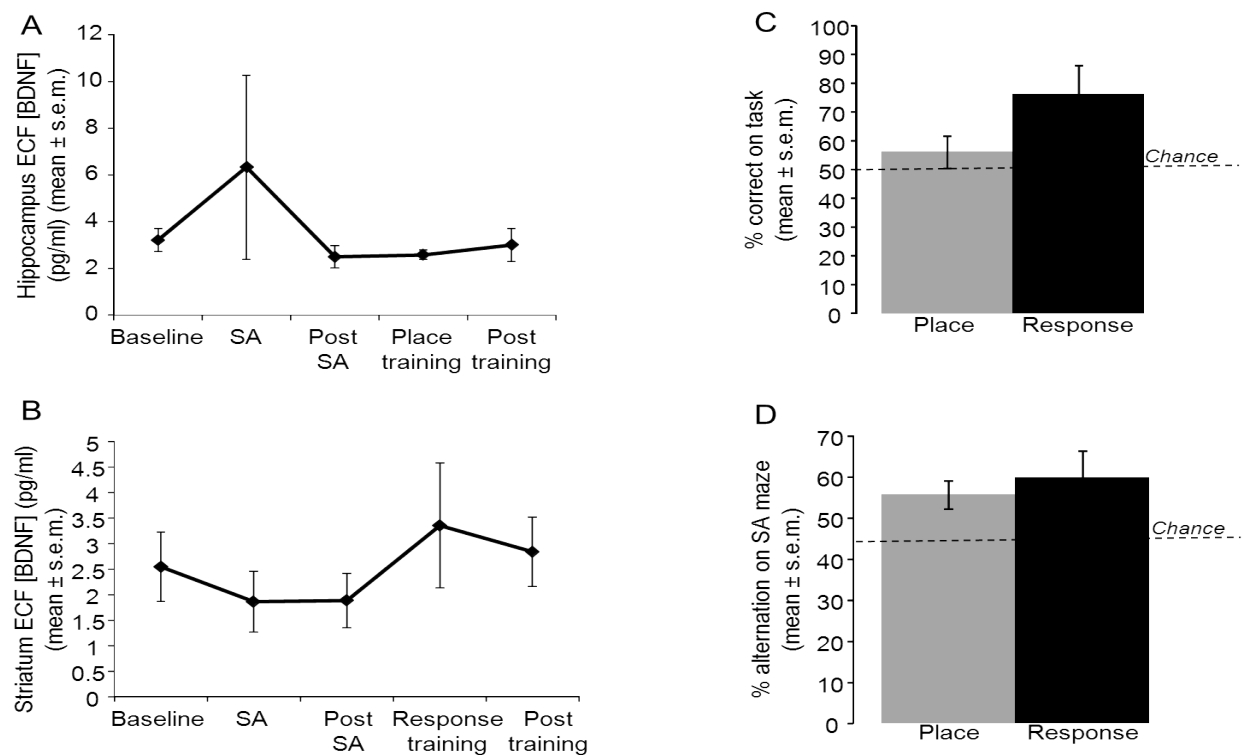


Figure 4.11. No change in ECF BDNF in the hippocampus or striatum during place or response learning in cognitively primed rats. A) Hippocampus ECF BDNF was not significantly different from baseline during SA testing or place training. B) Striatum ECF BDNF was not significantly different from baseline during SA testing or response training. C) Rats trained on the response task learned the task, as their scores were above chance (50%). Some rats that were trained on the place task learned the task while others had scores below 50%. D) During SA testing, both groups of rats that were place and response trained had normal alternation scores that were above chance (44%). N=5 for hippocampus, N=5 for striatum.

TRANSITIONAL THOUGHTS

Our findings from Chapter 4 showed that, compared to controls, physically active rats increase BDNF release in the hippocampus and striatum during task learning. Also, exercise rats that were the best place learners had higher extracellular BDNF in the hippocampus during place learning compared to the worst place learners. These findings support our theory of “Use it and Boost it”—physical activity engages the hippocampus and striatum, which increases BDNF release, which supports learning enhancements.

Cognitive priming did not change BDNF release in the hippocampus or striatum during place or response training, respectively. It is possible that there were methodological errors in this experiment that led to these findings. However, it is also likely that cognitive priming does not have an effect on BDNF release, but instead enhances learning by potentiating intracellular BDNF-TrkB signaling cascades. In Chapter 5, we measured changes in GSK3 β inhibition, which is downstream of BDNF-TrkB signaling. We hypothesized that rats that were cognitively primed would have higher GSK3 β inhibition in the hippocampus and striatum. Using brains collected from Chapter 3, we tested the effects of cognitive activity and BDNF-TrkB signaling on GSK3 β inhibition in the hippocampus and striatum.

CHAPTER 5:
GSK3 β INHIBITION IN THE HIPPOCAMPUS AND STRIATUM FOLLOWING PLACE
AND RESPONSE TRAINING IS NOT MODULATED BY BDNF-TRKB SIGNALING

ABSTRACT

BDNF signaling through TrkB receptors in the hippocampus and striatum modulates enhancements in place and response learning, respectively, produced by prior cognitive activity. However, the downstream targets of BDNF-TrkB signaling that may underlie cognitive activity-induced enhancements in learning remain unknown. Inhibition of glycogen synthase kinase 3 β (GSK3 β) may be an intermediary step in the regulation of learning, memory, and neural plasticity by BDNF. BDNF-TrkB signaling leads to inhibition of GSK3 β by its phosphorylation. Inhibition of GSK3 β has in turn been shown to enhance learning and memory in models of Alzheimer's disease. The current study investigates the relationship between BDNF-TrkB signaling, cognitive activity, and GSK3 β phosphorylation status in healthy young adult male rats. Phosphorylation status of GSK3 β and total GSK3 β in the hippocampus and striatum of rats were quantified via Western blots. Place and response training, but not SA testing, increased GSK3 β inhibition in both the hippocampus and striatum. We found no consistent effect of K252a and cognitive priming on GSK3 β inhibition in the hippocampus or striatum. It is possible that place- and response-training increased GSK3 β inhibition while SA testing did not because of differences in the task attributes. For example, SA testing, compared to place and response training, has no food reward, may have a lower cognitive demand, and lasts only 20 min instead of 1 hr. However, GSK3 β inhibition in the place and response trained rats, but not the SA tested rats may have been due the fact that SA-tested rats did not experience cannula surgery or

intracranial infusions. If the inhibition of GSK3 β were due to infusions *per se*, it is unlikely that increased inhibition would be observed in both the hippocampus and the striatum. Thus, while place and response training appear to inhibit GSK3 β , the results do not reveal a role for BDNF-TrkB signaling on GSK3 β inhibition in the hippocampus and striatum.

INTRODUCTION

Priming the brain with cognitive activity using a spatial working memory task such as spontaneous alternation (SA) enhances hippocampus-sensitive place and striatum-sensitive response learning (Chapter 3; Korol et al., 2013). This priming activity also increases brain-derived neurotrophic factor (BDNF) in the hippocampus and striatum. Limiting BDNF signaling through TrkB receptors either by sequestering BDNF or by blocking receptor activation attenuates learning and memory enhancements following exercise (Chapter 2; Vaynmann et al., 2004) and following priming with SA (Korol et al., 2013; Chapter 3). For example, intrahippocampal and intrastratial treatment with the TrkB inhibitor, K252a, attenuates SA-induced place and response learning enhancements, respectively (Korol et al., 2013; Chapter 3). While it is becoming well established that BDNF-TrkB signaling is involved in the neural plasticity underlying learning and memory (Lu, 2003; Tyler et al., 2002; Zeng et al., 2012), the signaling cascades downstream of BDNF-TrkB signaling mediating learning enhancements induced by prior cognitive activity remain relatively unknown.

BDNF-TrkB signaling initiates several different intracellular signaling pathways, all of which could support learning (see Korol et al., 2013; Figure 3.9). BDNF-TrkB signaling activates PI3K/Akt, which leads to glycogen synthase kinase 3 β (GSK3 β)

inhibition (Hoppe et al., 2013; Li et al., 2007). Like BDNF-TrkB signaling, GSK3 β inhibition is activity-dependent (Peineau et al., 2007; Hooper et al., 2007).

The role of GSK3 β activity in learning and memory is most often studied in transgenic animals and pathological states. In mouse models of Alzheimer's disease with hyperactive GSK3 β and in animals that over-express GSK3 β , drugs that inhibit GSK3 β activity ameliorate learning and memory deficits (del Ser et al., 2013; Engel et al., 2006; Liu et al., 2003). These findings suggest that GSK3 β activation may impair cognition and the corollary, that GSK3 β inhibition enhances cognition. However, it appears that some level of GSK3 β activity must be maintained for normal learning and memory; in healthy animals, drugs that chronically eliminate GSK3 β activity impair memory processing (Kimura et al., 2008). Therefore, a balance of GSK3 β activity and inhibition seems important for learning and memory in healthy animals.

In the current study, the effects of BDNF-TrkB signaling induced by cognitive activity on GSK3 β phosphorylation were examined in healthy young-adult male rats. Total and phosphorylated GSK3 β (at serine 9) in the hippocampus and striatum were quantified via Western blot. To examine task x brain area changes in GSK3 β , the hippocampus and striatum samples were harvested from rats that were untested and untrained, tested on an SA task, trained in place or response tasks, or primed with SA testing before place or response training (see Chapter 3). This multiple memory system approach has been applied successfully to identify biological bases of learning and memory processes across multiple measures (Chapter 4; Colombo et al., 2003; Pych et al., 2005; Chang and Gold 2003; Pisani et al., 2012). To test the role of BDNF-TrkB signaling in GSK3 β inhibition, separate groups of rats that were trained on place or

response tasks or primed with SA prior to place or response training, received intrahippocampal (for place-trained rats) or intrastratial (for response-trained rats) infusions of the TrkB inhibitor, K252a, or vehicle (aCSF+5%DMSO) 30 min before place or response training.

METHODS

Experiment summary

Rats underwent cannula surgery, were allowed a week to recover, and were subsequently placed on food restriction for approximately 7 days. One week following the start of food restriction, rats were either tested on a 4-arm maze for spontaneous alternation (SA) or left undisturbed in their home cages (untested controls). Thirty minutes after SA testing or in controls, thirty minutes prior to place or response learning, rats were infused with K252a or vehicle (artificial cerebrospinal fluid (aCSF) and 5% dimethyl sulfoxide (DMSO)). Thirty min following K252a or vehicle infusions into the hippocampus or striatum, rats were trained on a place or response task, respectively. Thus, there were two priming conditions (untested controls and SA-tested rats) and two drug treatment conditions (vehicle and K252a) generating four experimental groups for each of the two learning tasks: 1) unprimed + vehicle (N=7 for response, N=9 for place learning) or 2) unprimed + K252a (N=7 for response, N=7 for place learning), 3) SA-primed + vehicle (N=8 for response, N=9 for place learning) or 4) SA-primed + K252a (N=6 for response, N=8 for place learning). Rats were sacrificed immediately following training to analyze GSK3 β activation states in the hippocampus and striatum.

A subset of rats (N=5) was neither place nor response trained, but was instead tested for SA and euthanized 1 hr following SA testing to establish the baseline levels of GSK3 β activation in primed rats. A second subset of rats was food-restricted for 7 days but never tested on any of the behavioral paradigms (no SA testing, place, or response training) and was used as an untested but handled control (N=6). Hippocampi and striata were rapidly dissected and analyzed for GSK activation states via Western blots.

Cannula Implantation

Rats were anesthetized with 2-3.5% isoflurane and received 30,000 units of penicillin (i.m.; Dura-Pen; Henry Schein, Inc., Indianapolis, IN, USA) and injections of Rimadyl (5 mg/kg, s.c.) for analgesia prior to stereotaxic surgery. Sterile, stainless steel guide cannulae (22 gauge, 6 mm; Plastics One, Inc., Roanoke, VA, USA) aimed either at the dorsal hippocampus (AP -3.8, ML \pm 2.5, -1.9 mm ventral to dura) or the dorsal striatum (AP +0.2, \pm ML 3.6, -2.8 mm ventral to dura), were implanted bilaterally in all rats. Rats with cannula implanted in the hippocampus were place trained; rats that were implanted in the striatum were response trained. Coordinates were chosen based on reports demonstrating drug and hormone actions in the hippocampus and striatum on place and response learning (Zurkovsky et al., 2006, 2007, 2011; Newman et al., 2011) and were adapted from the atlas of Paxinos and Watson (1986). Four stainless steel screws were placed into the skull for anchors and the assemblage was cemented in place with dental acrylic. To keep cannulae open, 28 gauge stylets (Plastics One, Inc.) cut to the length of the guide cannulae were inserted at the time of surgery and were removed only during the central infusions.

Drug Infusion

Rats received K252a or vehicle infusions 30 min prior to training on place or response learning tasks. Bilateral infusions (0.5 μ l/side in 1 min) of K252a (25 μ M) or vehicle (5% DMSO and aCSF in mM: 128 NaCl, 2.5 KCl, 1.3 CaCl₂, 2.1 MgCl₂, 0.9 NaH₂PO₄, 2.0 Na₂HPO₄, 1.0 dextrose) were made into either the hippocampus or striatum. A CMA microdialysis infusion pump was used to deliver the K252a or vehicle through a 29-gauge needle attached to a 10 μ l Hamilton syringe. The needle was left in place for 1 min to allow diffusion away from the infusion site.

Spontaneous Alternation Maze Testing

SA-primed rats were placed on a 4-arm, plus-shaped maze (Figure 5.1A). The maze was located in the center of the 3.5 m x 3 m testing room on a table 76 cm above the floor surrounded by a rich assortment of extra-maze visual cues. During a testing session, the rat was placed in a start arm and allowed to explore the maze freely for 20 min while the number and sequence of arm entries were recorded. An alternation occurred when the rat entered all four arms within five consecutive arm entries. A moving window of five-arm-entries was analyzed for possible alternations across all arms entered by the rat within a single testing period. For example, entering arms ABDAC in five consecutive arm choices would be considered an alternation. However, entering arms ABACB in five consecutive arm choices would not be considered an alternation.

Place and Response Maze Environment

Place and response training were conducted in a 4-arm, plus-shaped maze configured into a “T” in which each trial started from either the north or south arm. For both tasks, the rats were trained to locate a food reward (half of a Frosted Cheerio ©) at the end of the goal arm.

The full length of each alleyway of the T-maze was approximately 104 cm. Each of the four arms of the maze was 13 cm wide, 18 cm high, and 46 cm in length. The center of the maze, from which all four arms extended, was 12 cm². The maze walls were constructed of black, 0.64-cm-thick Plexiglas® and the floor of black, matte Plexiglas®. The maze was affixed to a platform (46 cm in diameter) that allowed for the rotation of the maze. Plastic perforated food receptacles with inaccessible food reward were attached to the ends of each maze arm to discourage the use of odor cues to find the reward. A lamp with indirect lighting was placed in each corner of the room, providing symmetrical ambient light. A fan was used to mask building noise.

Place and Response Training Procedure

In the place task, rats learned the location of a food reward relative to the extramaze cues in the room (Figure 5.1B). Rats trained on the response task learned to locate a food reward using specific body turns (i.e. right or left turns; Figure 5.1C). In the response task, ceiling-to-floor curtains surrounded the maze to minimize extramaze cues. The presence or absence of extramaze cues respectively promotes the use of place or response strategies for efficient learning. However, the two versions of the task had the same locomotor and motivational requirements. For each rat, start arm positions were quasi-randomized across twenty trials, so that each start arm (north or

south) was used for 10 trials. For the place task, the goal location (east or west) was counterbalanced while for the response task the direction of body turn (right or left) was counterbalanced across rats within a treatment condition.

Training took place between 3 and 8 hr after the start of a light cycle. Rats were placed in a fresh, clean cage without food or water and moved into the training room for 15 minutes to allow acclimation to the maze environment prior to training.

A trial started when a rat was placed in the start arm and ended after all four paws entered a choice arm. For each trial, the rat was placed in a start arm and given the choice of going down one of the two possible reward arms. When the rat chose the rewarded arm, it was allowed to remain there until eating was complete before being returned to the holding cage for 30 sec before the next trial began. If the rat chose the incorrect arm, it was allowed to remain in that arm for approximately 5 sec before being returned to the holding cage for 30 sec before the next trial. The maximum time allotted for rats to choose an arm was 2 min, after which the rat was returned to the cage without making an arm choice. These omissions were not considered arm choices and did not contribute to the number of recorded correct or incorrect choices. Between trials, the maze was rotated 90° to reduce the use of intramaze cues to find the goal arm. All rats received 75 training trials. The learning criterion was 9 correct trials out of 10, with at least 6 consecutive correct trials.

Immediately after training or 1 hr after spontaneous alternation testing, rats were overdosed with sodium pentobarbital (75 mg/kg), after which they were decapitated and brain tissue was collected for biochemical assessment. Control rats that were not primed with SA were euthanized at times similar to those of rats tested on SA.

Post Sacrifice Tissue Preparation

Rats were anesthetized with a lethal injection of sodium pentobarbital (i.p. 75 mg/kg). Brains were dissected on ice and within 150 sec frozen on dry ice and stored at -80°C. The samples were mechanically homogenized on ice in a lysis buffer (50 mmol/L tricine, 0.1% β -mercaptoethanol, 10 mmol/L EDTA, 100 mmol/L NaF, 0.5 mmol/L PMSF and 1 L dH₂O), phosphatase inhibitors (Cocktail Set I, Calbiochem, La Jolla, CA) and dissolved protease inhibitor tablets (Complete Mini tablets, Roche Diagnostic Corporation, Indianapolis, IN). After homogenizing, the samples were transferred to a microfuge tube and centrifuged at 4°C for 15 min at 10,000 rpm. The supernatant was collected and stored in -80°C freezer until processed for western blots.

Protein assay

The protein concentrations of the samples were determined using a Micro-BCA assay kit (Pierce, Rockford, IL). Each tissue sample was diluted 1:25 with deionized water. Standards (25 μ l) ranging from 0 μ g/ml to 2000 μ g/ml, diluted samples (25 μ l), and lysis buffer controls (diluted 1:25, 25 μ l) were added in triplicate to a 96-well microtiter plate. A working reagent (100 μ l) was added to each well and incubated at 37°C for 30 min. After incubation, the plate was cooled to room temperature (25°C). Absorbance at 570 nm was then measured in a ThermoMax® microplate reader with Ascent software (Fischer Scientific, Pittsburgh, PA).

Western blot sample preparation

Samples for western blots were prepared so they contained a final protein concentration of 1 μ g protein per μ l of solution and 5% β -mercaptoethanol. The calculated amount of brain supernatant was added to lysis buffer containing fresh

protease inhibitor tablets and phosphatase inhibitor 1:1 with 2X protein loading buffer (LI-COR Biosciences, Lincoln, NE) and 10% β -mercaptoethanol. Then the sample was flash frozen on dry ice, boiled for 10 min, and stored at -80°C until it was loaded onto a gel.

Western blots

To generate standard curves that allow comparisons across gels, samples from pooled hippocampal or striatal homogenates containing 10, 20, and 30 μ g total protein were loaded onto each gel. Standard curve samples, prepared experimental samples (20 μ g), and Kaleidoscope precision plus protein standards (3 μ l; BioRad Laboratories, Hercules, CA) were loaded onto a 10% SDS-polyacrylamide gel and resolved via electrophoresis for 75 min at 175 V. Following electrophoresis, proteins were transferred to a PVDF membrane (Immobilon-FL, Millipore, Billerica, MA) at 100V, for 2 hr at 4°C. Following protein transfer, membranes were blocked for 1 hr using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) diluted 1:1 with 0.05 M PBS. The membrane was then incubated at 4°C for 22 hr in anti-GSK3 β rabbit monoclonal antibody (1:1000; #9315, Cell Signaling, Rockford, IL) or anti-phospho GSK3 β (Ser9) rabbit monoclonal antibody (1:500; #9323, Cell Signaling, Rockford, IL), and anti- α -tubulin mouse monoclonal antibody (1:30,000; #T9026, Sigma-Aldrich, St Louis, MO) in 1:1 LICOR blocking buffer and 0.05 M PBS and 0.1% Tween 20 (Fischer Scientific, Fair Lawn, NJ). Following primary antibody incubation, the PVDF membrane was washed 4 times for 10 min in 0.05 M PBS and 0.1% Tween 20. Then the PVDF membrane was incubated for 1 hr at room temperature with IRDye 800 CW goat anti-rabbit (1:5000; LI-COR Biosciences, Lincoln, NE) and IRDye 680 LT goat anti-mouse (1:20000; LI-COR

Biosciences, Lincoln, NE) in 1:1 LICOR blocking buffer and 0.05 M PBS and 0.1% Tween 20. The membrane was then washed 4 times in 0.05 M PBS and 0.1% Tween 20, rinsed for 2 min in DI H₂O and dried overnight. Dry membranes were scanned on the Odyssey Classic Infrared imaging system (LI-COR Biosciences, Lincoln, NE) to quantify the protein bands.

Image analysis

Images of the western blots were analyzed using Odyssey software (LI-COR Biosciences). Each band was identified and manually fitted to measure integrated intensity of the band (band intensity X band area). In calculating integrated intensity, the Odyssey software automatically corrects for background staining using the average background of the pixels surrounding the band of interest. The Odyssey software performs background correction by finding the minimum intensity value and subtracting the average of that value from all of the pixels in the protein band. Standard curves (Figure 5.2A and 5.2B) included on each blot were used to determine linearity of the blots and the relative amounts of pGSK3 β and GSK3 β present in each sample band. Values from the 20 μ g sample from the standard curve on each blot were calculated and divided by values for α -tubulin (loading control). For each blot, protein values of our experimental samples were first standardized to values for α -tubulin and then were calculated as a percentage of the 20 μ g standard for presentation. This approach allowed us to account for differences in loading within a blot (α -tubulin standardization) and for between-blot differences in immunoblotting properties (20 μ g-sample standardization).

Each sample was run in triplicate on different blots and standardized to its α -tubulin control. The median value from each set of triplicates was used to generate the percentage of the 20 μ g standard that was used for group calculations. The means of these median values were calculated to generate the percent of 20 μ g standard values for each treatment group. The ratio of pGSK3 β to GSK3 β was determined to generate a measure of GSK3 β inhibition. There are two isoforms of GSK3 β (Mukai et al., 2002). Although both isoforms of phosphorylated GSK3 β appeared on our blots, only type 1 GSK3 β was analyzed due to its consistent presence in our samples with consistently linear standard curves.

Statistical Analysis

To test for differences in pGSK3 β :GSK3 β in the hippocampus and striatum across SA-tested and untested controls, training groups, and drug treatments one-way analyses of variance (ANOVAs) were performed. Also, two-way ANOVAs were conducted to test for the main effects of priming (SA-tested or untested controls), main effects of drug (K252a or vehicle), and their interaction on pGSK3 β :GSK3 β in the hippocampus and striatum of place- and response-trained rats.

RESULTS

pGSK3 β :GSK3 β levels were increased in the hippocampus and striatum following place and response training compared to SA tested and untested, untrained controls. There was no effect of SA testing on pGSK3 β :GSK3 β levels in the hippocampus or striatum compared to untested untrained control rats.

pGSK3 β :GSK3 β levels in the hippocampus were significantly increased in place-trained rats compared to rats that were untested or had only SA testing ($F_{(5,38)}=2.6$,

$p < 0.05$; Figure 5.3A). There were no significant or consistent changes in total GSK3 β in either the hippocampus or striatum after training on either task. Therefore, the increases relative to the controls are in the phosphorylation state of GSK3 β , with that phosphorylation thought to indicate inhibition. There was no effect of drug ($F_{(1,29)} = 0.24$, $p > 0.2$) or priming ($F_{(1,29)} = 0.415$, $p > 0.2$) on pGSK3 β :GSK3 β levels in the hippocampus of place trained rats. Also, there was no interaction ($F_{(1,29)} = 0.72$, $p > 0.2$) of cognitive priming and drug treatment on pGSK3 β :GSK3 β levels in the hippocampus of place trained rats. In the striatum of place-trained rats there was an increase in pGSK3 β :GSK3 β levels compared to SA tested and untested control rats, but this effect was not statistically significant ($F_{(5,38)} = 1.8$, $p > 0.1$; Figure 5.3B). There was no effect of drug ($F_{(1,29)} = 0.192$, $p > 0.2$) or priming ($F_{(1,29)} = 0.428$, $p > 0.2$) on pGSK3 β :GSK3 β levels in the striatum of place trained rats. Also, there was no interaction ($F_{(1,29)} = 0.164$, $p > 0.2$) of cognitive priming and drug treatment on pGSK3 β :GSK3 β levels in the striatum of place trained rats. However, post hoc t -tests revealed that rats that were unprimed and K252a-treated ($t_{11} = 3.4$, $p < 0.1$) and rats that were primed and vehicle-treated ($t_{13} = 2.6$, $p < 0.05$) prior to place learning had statistically higher levels of pGSK3 β :GSK3 β levels in the striatum compared to untested untrained controls.

In the hippocampus of response-trained rats, there was no significant effect of training or drug treatment on pGSK3 β :GSK3 β levels compared to SA-tested and untested control rats ($F_{(5,32)} = 1.5$, $p > 0.2$; Figure 5.4A). There was no effect of drug ($F_{(1,22)} = 0.84$, $p > 0.2$), no effect of priming ($F_{(1,22)} = 0.029$, $p > 0.2$), and no interaction of priming and drug ($F_{(1,22)} = 0.045$, $p > 0.2$) on pGSK3 β :GSK3 β levels in the hippocampus. Post hoc t -tests revealed that pGSK3 β :GSK3 β levels in the hippocampus of response-

trained rats that were unprimed and vehicle-treated ($t_{11}=1.7$, $p<0.1$), unprimed and K252a-treated ($t_{11}=1.8$, $p<0.1$), primed and vehicle-treated ($t_{11}=2.13$, $p<0.1$), and primed and K252a-treated ($t_{10}=2.28$, $p<0.05$) were increased compared to untested untrained controls. pGSK3 β :GSK3 β in the striatum was significantly increased in response-trained rats ($F_{(5,33)}=2.9$, $p<0.05$; Figure 5.4B) compared to rats that were untested controls. Two-way ANOVAs revealed that there was no effect of drug ($F_{(1,24)}=0.13$, $p>0.2$) or priming ($F_{(1,24)}=3.78$, $p<0.1$) on pGSK3 β :GSK3 β levels. Also, there was no interaction ($F_{(1,24)}=1.79$, $p>0.2$) of cognitive priming and drug treatment on pGSK3 β :GSK3 β levels in the striatum of place trained rats.

Since the pGSK3 β :GSK3 β levels are made up of the measurements of pGSK3 β and total GSK3 β , these data are included in Figures 5.5 and 5.6. For place-trained rats pGSK3 β and total GSK3 β levels of the hippocampus are found in Figures 5.5A and 5.5B and the striatum are found in Figure 5.5C and 5.5D. For response-trained rats pGSK3 β and total GSK3 β levels of the hippocampus are found in Figures 5.6A and 5.6B and the striatum are found in Figure 5.6C and 5.6D.

DISCUSSION

Training in the place and response tasks increased GSK3 β inhibition (i.e. the pGSK3 β :GSK3 β ratio) in the hippocampus and striatum compared to control conditions, including rats with no behavioral tests or SA only. Furthermore, we were surprised to find that treatment with K252a failed to modulate relative levels of phosphorylation of GSK3 β in any consistent manner, given our behavioral results showing an important role for BDNF-TrkB signaling following cognitive priming (Chapter 3). Thus, we were unable to demonstrate a direct link between BDNF-TrkB signaling and GSK3 β inhibition

in the hippocampus and striatum. Also, we were surprised to see a lack of dissociation between the pGSK3 β :GSK3 β levels in the hippocampus and striatum following place and response learning. Instead, we found that GSK3 β inhibition in the hippocampus and striatum is not sensitive to engagement in canonical strategies, but was more generally sensitive to the learning experience itself.

GSK3 β inhibition was also similar between untested/untrained control rats and rats tested on SA only with no further training. While these results suggest that SA testing alone does not have an effect on GSK3 β inhibition, our findings from Chapter 3 show that SA testing alone is a robust enough cognitive activity to increase protein levels of mBDNF in the hippocampus and striatum. Furthermore, we found that priming with SA may increase BDNF-TrkB signaling that in turn produces enhancements in place and response learning. Given that we did not see any effect of blockade of BDNF-TrkB signaling with K252a treatment on GSK3 β inhibition, it is possible that the SA-induced increases in BDNF content are not robust enough to modulate GSK3 β inhibition in the hippocampus and striatum. It is possible that the priming effect of SA testing on BDNF-TrkB signaling during place and response tasks is potentiated during the training period. Thus, if we put SA tested rats on the maze 1 hr after testing for just one trial to potentiate BDNF-TrkB signaling, we maybe would have seen an effect of SA testing on GSK3 β inhibition. However, given our findings that blockade of BDNF-TrkB signaling had no effect on GSK3 β inhibition it seems unlikely that potentiating BDNF-TrkB signaling in SA-tested rats would modulate GSK3 β inhibition in the hippocampus and striatum.

The fact that GSK3 β inhibition was increased with place and response learning but not SA testing points to differences in task attributes between SA testing and place and response learning. For instance, SA testing has no food reward, lasts only 20 min, and may require less cognitive resources. Given that the rats engaged in the place and response tasks receive a food reward that is likely to be arousing on average once every minute, for 60 min, and do require significant resources from the hippocampus and striatum (Newman et al., in prep; Gold et al., 2013), it is likely that the place and response tasks would induce higher levels of GSK3 β inhibition in the hippocampus and striatum. However, it is also possible that limitations in our experimental design led to increases in GSK3 β inhibition in the hippocampus and striatum of place- and response-trained rats and not SA-tested rats. Perhaps, the increases in GSK3 β inhibition in place- and response-trained rats were from cannula surgery and/or intracerebral infusions of K252a or vehicle, which SA-tested rats did not receive. Thus, we cannot definitively say that the GSK3 β inhibition observed in place- and response-trained rats is due to the training experience in itself. However, it seems unlikely that the cannula surgery/ drug infusions caused the increase in GSK3 β inhibition given that the noncannulated brain regions also showed increases in GSK3 β inhibition following place and response training. Future studies are underway to test possible effects of cannula surgery and intracerebral infusions on GSK3 β inhibition.

In place-trained rats, primed K252a-treated rats had slightly decreased GSK3 β inhibition in the hippocampus compared to the primed vehicle-treated groups, but this effect was not statistically significant. However, further examination of the pGSK3 β and total GSK3 β levels suggests that the shift in the pGSK3 β :GSK3 β levels are due to, if

anything, a decrease in total GSK3 β in primed vehicle-treated rats. It is possible that the BDNF-TrkB signaling induced by SA testing and place training triggered GSK3 β degradation. Previous studies have demonstrated that GSK3 β phosphorylation—initiated by Akt signaling—leads to its degradation (Failor et al., 2007). Given that BDNF-TrkB signaling activates PI3K/Akt signaling (Almeida et al., 2005; Yamada et al., 1997; Yamada et al., 2001) leading to GSK3 β phosphorylation at serine 9 it is possible that with significant amounts of cognitive activity, BDNF-TrkB signaling can lead to sufficient phosphorylation of GSK3 β to induce GSK3 β degradation. For the striatum, we saw no effect of priming on total GSK3 β levels in response-trained rats. In addition, we saw no effect of response training on total GSK3 β levels in the striatum. Thus, it is possible that GSK3 β degradation in the striatum is not sensitive to cognitive activity.

In response-trained rats, K252a treatment increased GSK3 β inhibition in primed, but not in unprimed rats, however this difference was not significant. Regardless, this finding was surprising as we found in Chapter 3, that K252a-treated unprimed rats learned the response task faster than vehicle-treated controls and K252a-treated primed rats. These data suggest that response learning enhancements observed in unprimed rats administered intrastriatal K252a are not mediated by GSK3 β inhibition in the striatum. Previous studies have demonstrated that K252a treatment, in conditions that lack BDNF signaling, activates PI3K/Akt signaling cascades, which are upstream of GSK3 β inhibition (Roux et al., 2002). Thus, we had hypothesized that the response learning enhancements in K252a-treated unprimed rats were possibly due to increased striatal GSK3 β inhibition. However, given the current data showing no increased inhibition, K252a treatment must act through other signaling cascades to enhance

learning in unprimed rats. Also, further analysis of the pGSK3 β and total GSK3 β graphs show that unprimed K252a-treated rats had lower levels of pGSK3 β and higher levels of total GSK3 β compared to primed K252a-treated rats. Thus, the difference in GSK3 β inhibition between primed and unprimed K252a-treated rats is likely due to a difference in pGSK3 β and total GSK3 β protein content.

GSK3 β inhibition in the hippocampus and striatum did not dissociate by task. Instead, in the place and response tasks, both the hippocampus and striatum had increased GSK3 β inhibition compared to controls. Previously others have reported an increase in immediate early gene activation compared to controls but a lack of dissociation between place and response learners (Gill et al., 2007). Furthermore, the hippocampus and striatum both increase neural activity during both place and response training, responding to both movement and spatial information (Yeshenko et al., 2004). Because GSK3 β inhibition is activity dependent, it is possible that it does not discriminate between relative activity of the hippocampus and striatum, but instead responds to activity in general. However, given that we did not see increases in GSK3 β inhibition in the hippocampus and striatum following SA testing, GSK3 β inhibition may require a certain threshold of activity and/or a more prolonged period of activity. It has been shown that prolonged exercise, a highly stimulating experience, requiring high neural activity (Hirase et al., 1999) also increased GSK3 β inhibition in the hippocampus (Bayod et al., 2011; 2014). Our findings demonstrate that GSK3 β inhibition in the hippocampus and striatum is sensitive to highly stimulating experiences that require high cognitive resources, such as place and response learning.

Past studies have shown that the use of the multiple memory systems approach, examining both the hippocampus and striatum during and/or following place and response training, is informative in determining the role of neurochemical and cell signaling changes that support learning in the hippocampus and striatum. For example, cyclic AMP response element binding protein phosphorylation (pCREB) and c-Fos induction in the hippocampus and striatum reflected place or response strategy selection in the ambiguous T-maze (Colombo et al., 2003). In Colombo et al., 2003, rats that used response (striatum-sensitive) strategies had increased pCREB in the striatum and decreased pCREB and c-FOS in the hippocampus, while rats that used place (hippocampus-sensitive) strategies had increased pCREB and c-Fos in the hippocampus and decreased pCREB in the striatum. Of note, the levels of pCREB in the hippocampus and striatum immediately following training were not different. Instead, a 1 hr wait was required to show the dissociation of pCREB in the hippocampus and striatum of rats engaging in place or response strategies. It is possible that if we had waited 1 hr following training we would have seen the lasting dissociable changes in GSK3 β inhibition in the hippocampus and striatum following place and response training. Like transcription factor activation, we expected to see brain region- and task-specific GSK3 β inhibition in the hippocampus and striatum following place and response learning. However, we were unable to identify GSK3 β inhibition in the hippocampus and striatum as a reliable biochemical marker of learning canonical tasks. Instead, we found that place and response training increased GSK3 β inhibition in both the hippocampus and striatum. In Chapters 2 and 3 we found that priming with exercise or SA testing increased BDNF protein in the hippocampus and striatum, in addition to

enhancing both place and response learning. The findings in the current study agree with the findings from Chapters 2 and 3 pointing to the collaboration, not the competition, of multiple memory systems.

BDNF-TrkB signaling did not modulate GSK3 β inhibition in the hippocampus or striatum. It is possible that other modulators of GSK3 β inhibition, such as Wnt signaling (Valvezan and Klein, 2012; Budnik and Salinas, 2011) are involved in increasing GSK3 β inhibition in the hippocampus and striatum of primed K252a-treated rats. Furthermore, there are two isoforms of GSK3 β . The two isoforms appear to have different kinase activity and maintain different distribution in neural cells (Mukai et al., 2002). For example, type 1 GSK3 β is mainly found in neuronal processes while type 2 is found in the soma, with lower kinase activity towards its substrate tau (Mukai et al., 2002). Thus, it is possible that GSK3 β type 2 regulates different substrates than does type 1. In many of our blots we did see a doublet for phosphorylated GSK3 β . However, we did not analyze the type 2 isoform because its protein bands were much less intense and the standard curve in the lower range (10 μ g) was not always visible. It is possible that we may have seen differences in type 2 GSK3 β inhibition due to place or response training, priming, or K252a treatment. Currently there are no reports dissociating the effects of type 1 vs type 2 GSK3 β activity on learning. Thus, it would be interesting to examine if type 2 GSK3 β in the hippocampus and striatum is inhibited following place and response training and/or SA testing.

Taken together, the current findings show that hippocampus- and striatum-sensitive tasks that are enhanced by BDNF-TrkB signaling also increase GSK3 β inhibition. However, because blockade of BDNF-TrkB signaling with K252a treatment

did not have any effect on GSK3 β inhibition, it is unlikely that the place and response training-induced increases in GSK3 β inhibition are due to BDNF-TrkB signaling. Further supporting this claim, there was no increase in GSK3 β inhibition following SA testing, which has been shown in Chapter 3 to increase BDNF in the hippocampus and striatum. Thus, GSK3 β inhibition may not be necessary/important for the effects of priming in the hippocampus and striatum. However, given the findings that BDNF-TrkB signaling is important for priming, it is time to move on to other downstream signaling pathways. Some prominent ones (ERK, PLC γ , PI3K/Akt) have also themselves been related to learning, though not with priming and not in a multiple memory system manner. The literature showing that GSK3 β inhibition plays a role in pathological models of learning and memory suggests that GSK3 β inhibition is important for learning in many contexts, but not in the priming effects induced by SA testing.

FIGURES

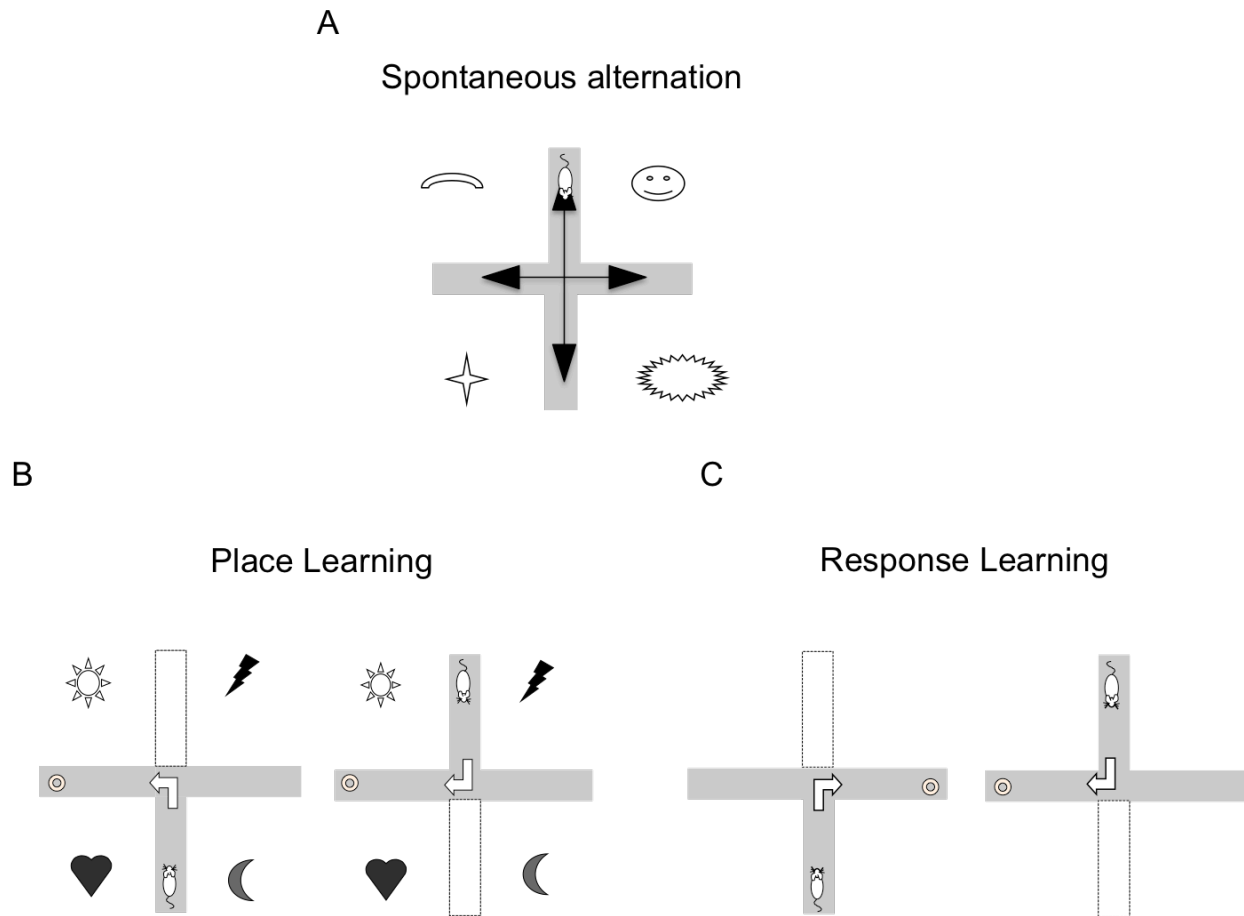


Figure 5.1. Spontaneous alternation and place and response tasks. In A) the 4-arm spontaneous alternation task, rats are given 20 min to explore all 4 arms of the maze and are tested on their working memory capacity by counting how many times the rat enters all 4 arms within 5 arm entries. In B) the place task, the rat learns the location of its food reward using the extramaze cues in the room. The place task requires intact hippocampal functioning for successful learning. The response training task C) requires the rat to learn the location of its food reward using egocentric body movements (i.e. left or right turns). The response task requires intact striatal functioning for successful learning.

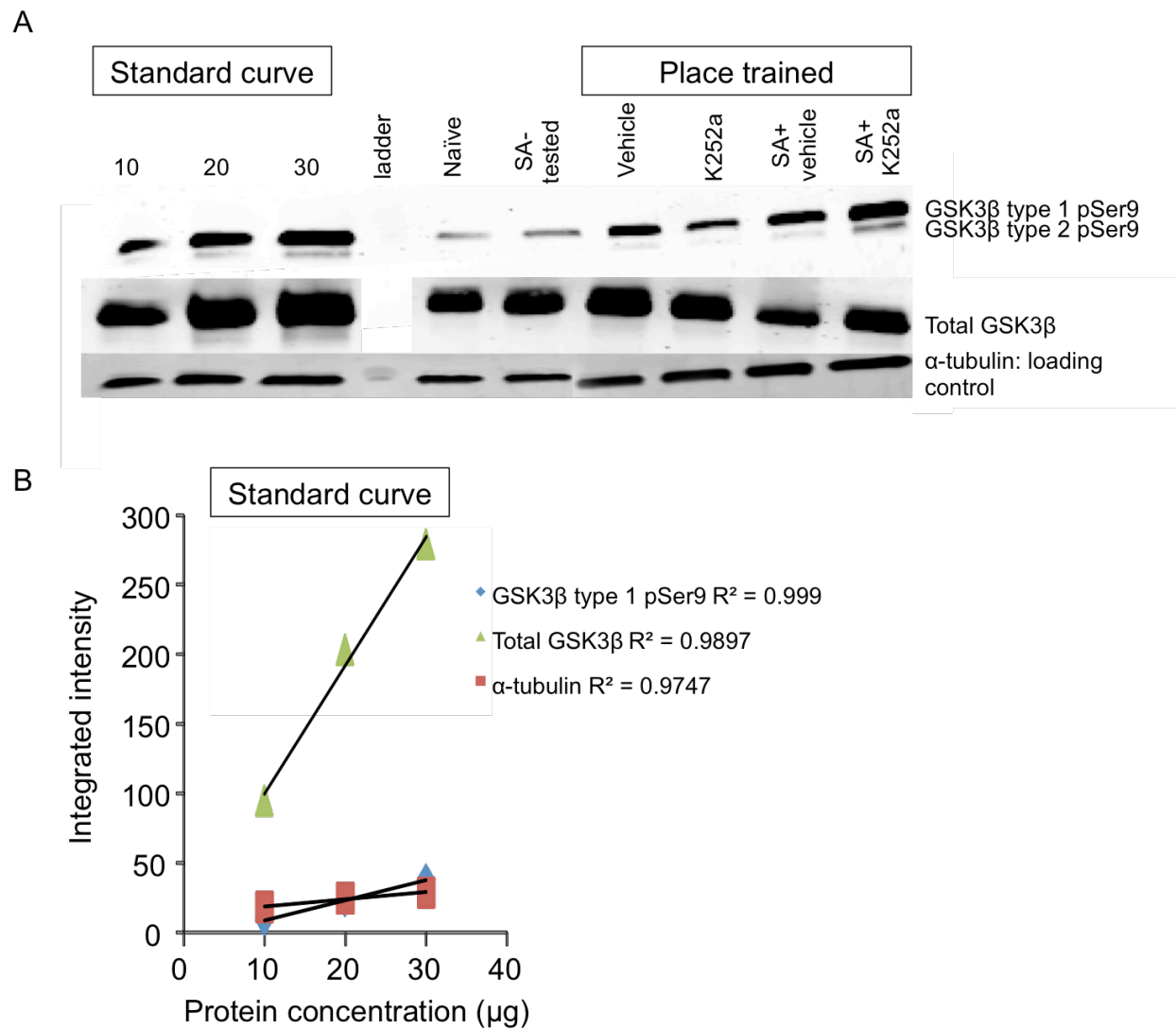


Figure 5.2. Representative western blots and standard curves of hippocampal homogenate. A) Representative Western blots of pGSK3β, total GSK3β, and loading control α-tubulin. The first 3 lanes are pooled hippocampi homogenate generating a standard curve. The last 4 lanes are place-trained rats. Pooled hippocampal homogenates were added in known protein concentrations and were calculated using Odyssey software to generate a B) standard curve.

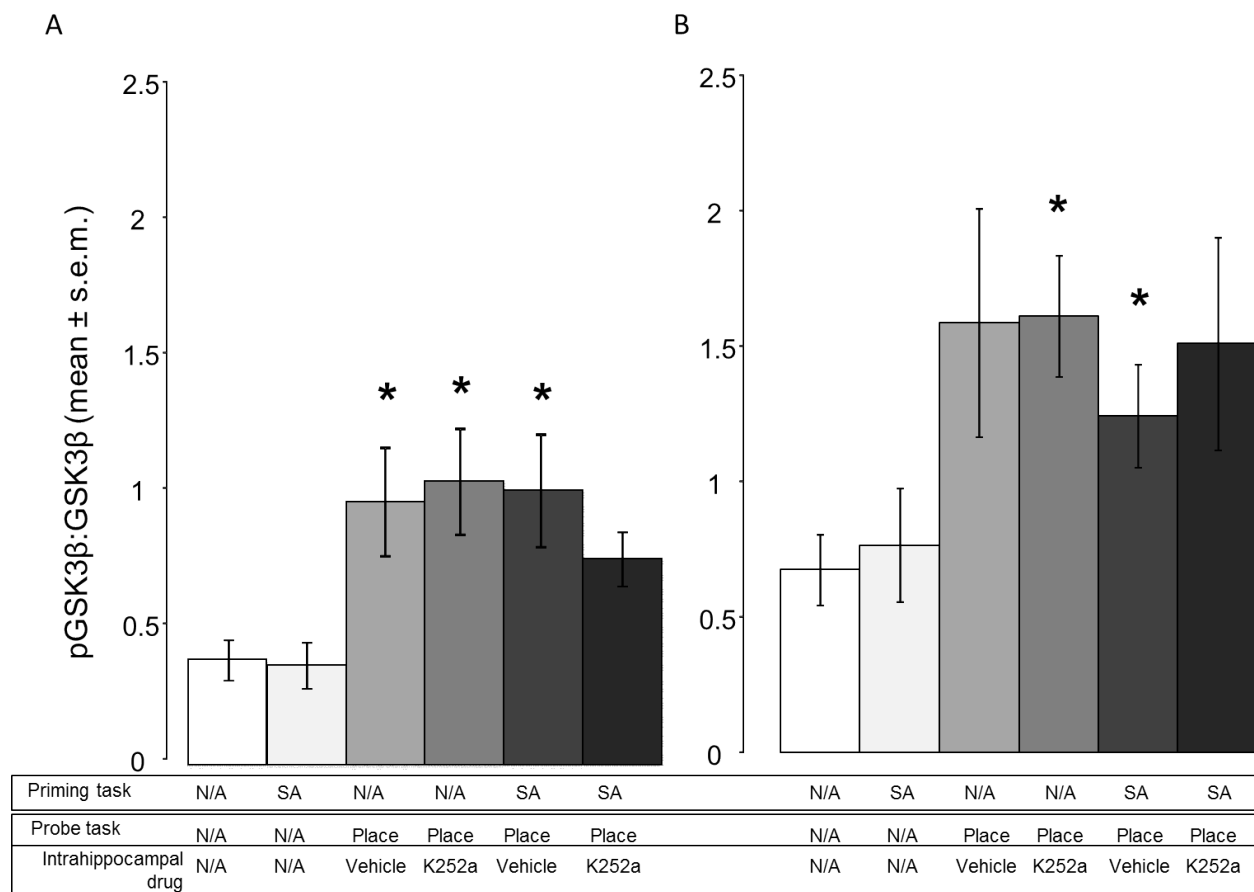


Figure 5.3. Place training increased pGSK3β:GSK3β levels in the hippocampus

and striatum. One-way ANOVAs revealed that compared to naïve and SA-tested rats, rats that were place trained had increased levels of pGSK3β:GSK3β in the A) hippocampus and B) striatum. Prior cognitive priming with SA testing and intrahippocampal K252a infusions did not have any effect on pGSK3β levels in place-trained rats. *=p<0.05 vs. SA tested and untested controls. N=6 for untrained untested controls, N=5 for SA tested controls, N=7 for unprimed vehicle, N=7 for unprimed K252a, N=8 for primed vehicle, N=7 for primed K252a.

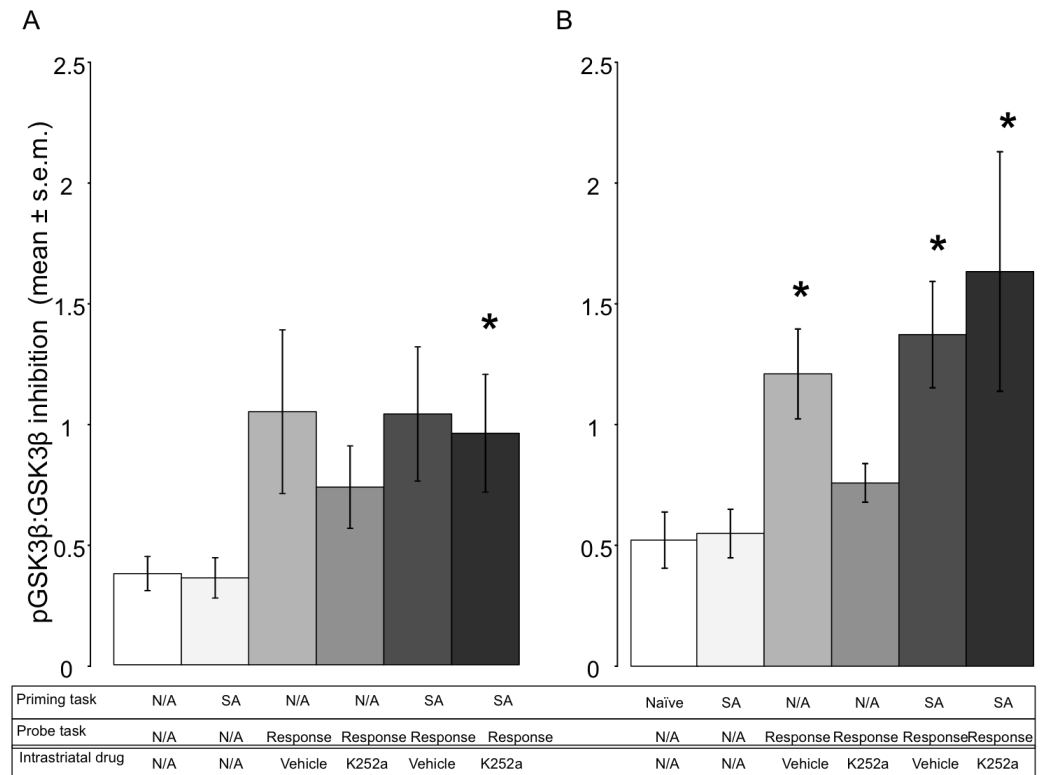


Figure 5.4. Response training increased pGSK3β:GSK3β levels in the hippocampus and striatum. One-way ANOVAs revealed that Compared to naïve and SA-tested rats, rats that were response trained had increased levels of pGSK3β:GSK3β in the A) hippocampus and B) striatum. Primed rats that were infused with K252a had higher levels of pGSK3β:GSK3β in the striatum than unprimed rats infused with K252a, but this effect was not statistically significant . *=p<0.05 vs. SA tested and untested controls. p=0.061 for striatum unprimed K252a vs. primed K252a. N=6 for untrained untested controls, N=5 for SA tested controls, N=8 for unprimed vehicle, N=7 for unprimed K252a, N=8 for primed vehicle, N=6 for primed K252a.

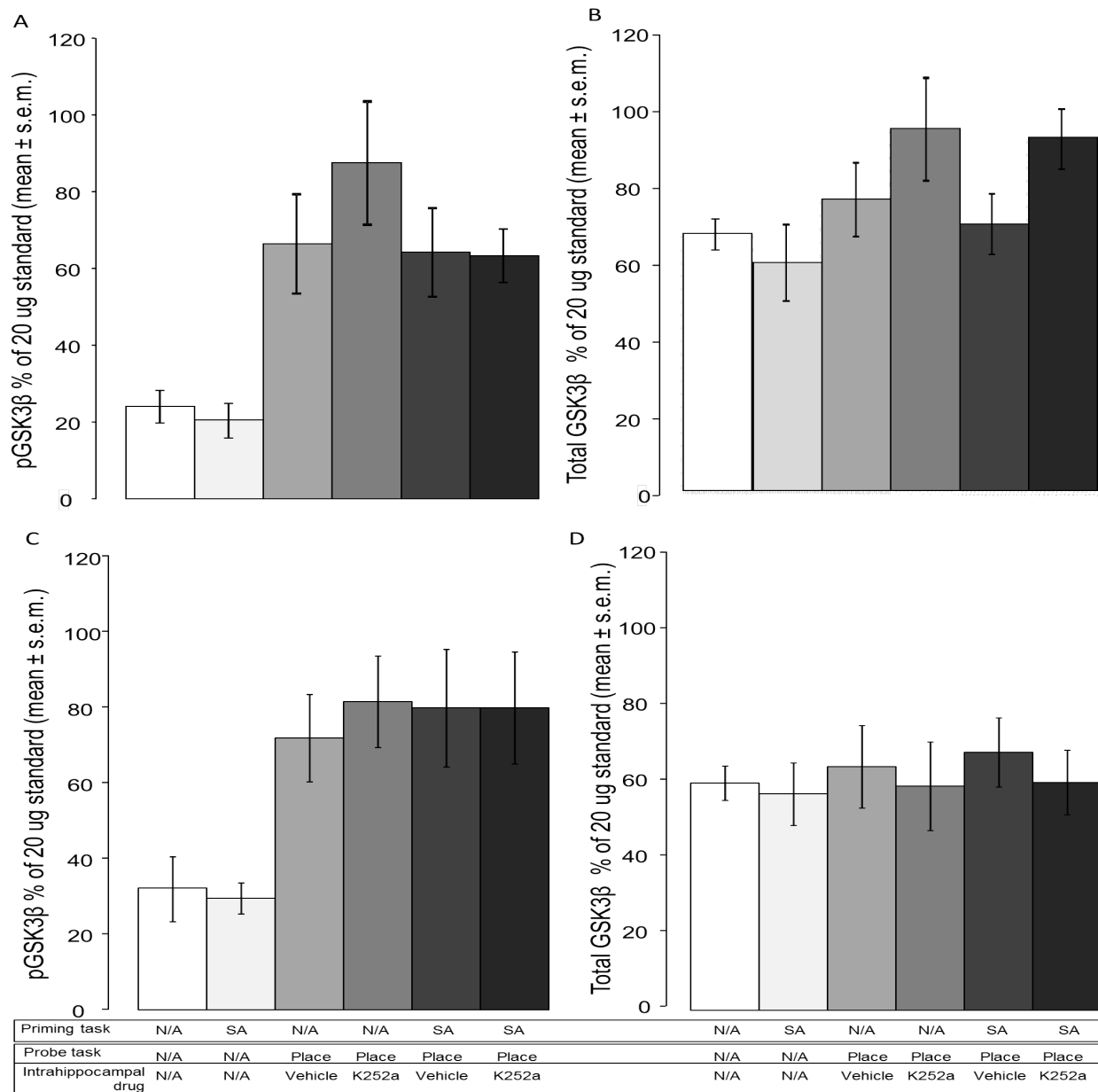


Figure 5.5. pGSK3β and total GSK3β levels in the hippocampus and striatum of place trained rats. In A and B) the hippocampus of place trained rats A) pGSK3β levels were increased and B) total GSK3β levels were not different compared to SA-tested or untested controls. In C and D) the striatum of place trained rats C) pGSK3β levels were increased and D) total GSK3β levels were not different compared to SA-tested or untested controls.

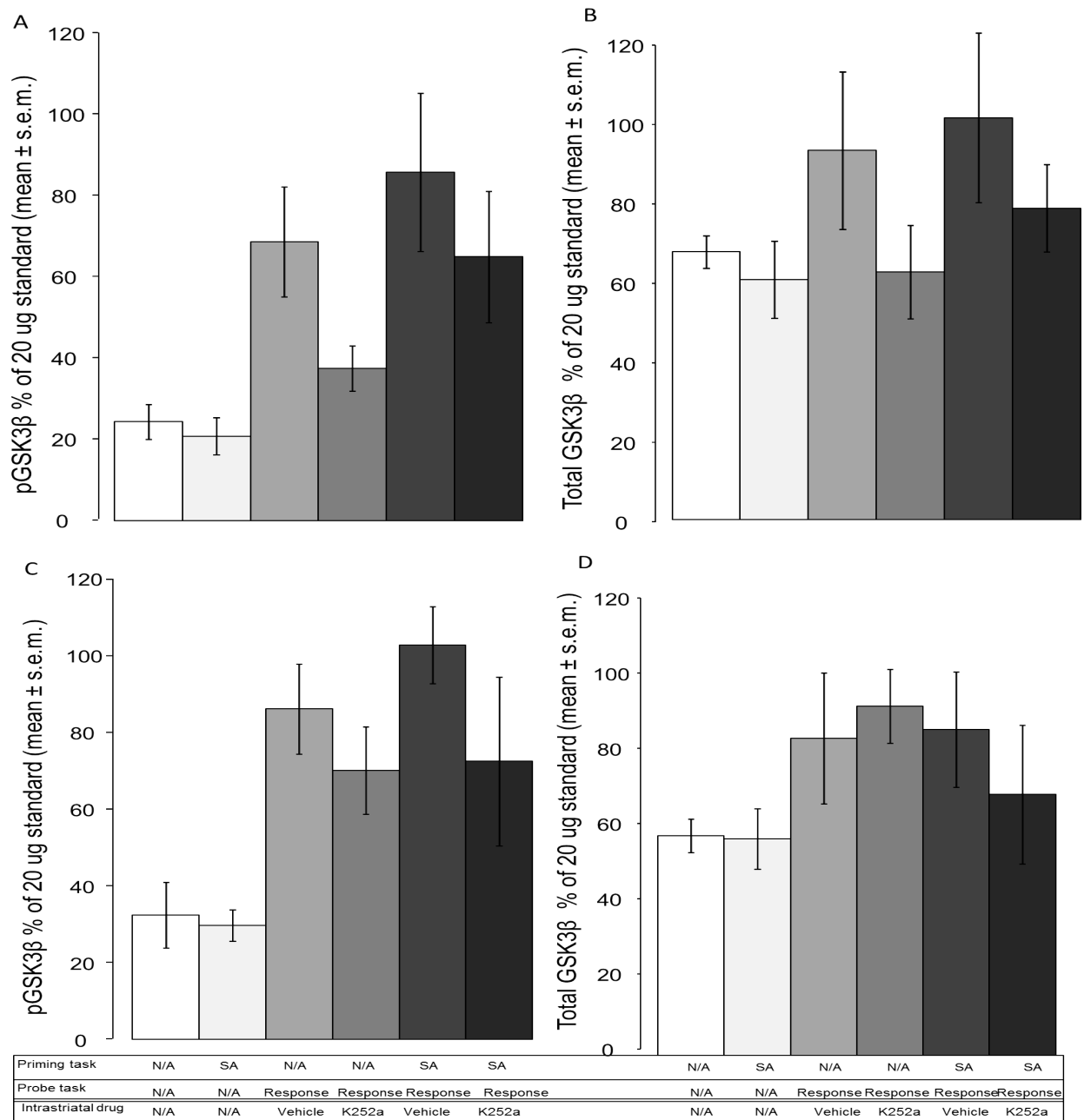


Figure 5.6. pGSK3β and total GSK3β levels in the hippocampus and striatum of response trained rats. In A and B) the hippocampus of response trained rats A) pGSK3β levels were increased and B) total GSK3β levels were not different compared to SA-tested or untested controls. In C and D) the striatum of response trained rats C) pGSK3β levels were increased and D) total GSK3β levels were not different compared to SA-tested or untested controls.

CHAPTER 6: USE IT AND BOOST IT: WRAPPING IT UP

Through the preceding series of studies, we have attempted to uncover the mechanisms by which cognitive and physical activity boost learning. In Chapters 2 and 3, rats that were physically active for 3 weeks or engaged in a spatial working memory task for 20 min learned both the place and response tasks more quickly than controls. To investigate the mechanisms underlying the activity-induced learning enhancements we examined the hippocampus and striatum because they are the neural systems involved in place and response learning, respectively. BDNF signaling in the hippocampus and striatum was a targeted mechanism because earlier studies have shown that BDNF gene expression and secretion are activity-dependent processes (Kesslak et al., 1998; Zafra et al., 1990; Hartmann et al., 2001; Balkowiec and Katz, 2002). Apart from being an activity-dependent protein, BDNF is also important for learning and memory processes (for review see Tyler et al., 2002; Cunha et al., 2010). Chapters 2 and 3 further confirmed that BDNF protein levels in the hippocampus and striatum increase following cognitive and physical activity. However, in Chapter 3 increases in BDNF protein did not necessarily equate to enhanced learning. At 24 hr following SA testing, BDNF levels in the striatum were robustly increased compared to controls even though there was no significant difference in response learning at this time point. Nonetheless, an increase in BDNF protein is likely a downstream effect of BDNF-TrkB signaling. Given that BDNF-TrkB signaling is activity-dependent and promotes learning and memory processes it is likely that cognitively and physically active animals rely on BDNF-TrkB signaling to support enhancements in learning. Our data showed that treatment with K252a, an inhibitor of TrkB, attenuated learning

enhancements induced by cognitive and physical activity. In control rats, K252a treatment enhanced response learning. Admittedly, K252a is not the most specific method for inhibiting TrkB signaling, and likely its non-specific effects lead to response learning enhancements in control rats. However, current methods that do specifically block TrkB signaling require longer treatment regimens (on the order of days), thus compromising our experimental design; that is, with longer treatment we are unable to block TrkB signaling after the priming and just prior to tests of subsequent learning. Other more specific pharmacological TrkB inhibitors available, such as AZ623, have yet to be tested for their efficacy over short exposure periods (Zage et al., 2011). For future studies testing the rapid effects of BDNF-TrkB signaling it would be worth using different specific, selective, and non-competitive TrkB inhibitors. Our attempts to further investigate K252a in the hippocampus and striatum of control rats were carried out in Chapter 5. Following the finding that BDNF-TrkB signaling contributes to activity-induced learning enhancements, the research direction focused on two possible candidate mechanisms for cognitive and physical activity-induced increases in BDNF-TrkB signaling: 1) increased BDNF release, leading to increased BDNF-TrkB signaling (tested in Chapter 4) and/or 2) potentiated intracellular BDNF-TrkB-initiated signaling cascades (tested in Chapter 5). Microdialysis measured extracellular levels of BDNF in the hippocampus and striatum before, during, and following learning. In sedentary control rats, dynamic release of BDNF was task- and brain region-specific. Rats engaged in the place task increased BDNF release in the hippocampus and decreased BDNF release in the striatum, while rats engaged in the response task showed the opposite effect of increased BDNF release in the striatum and decreased release in the

hippocampus. Compared to sedentary controls, physically active rats increased BDNF release in the hippocampus during place learning and increased BDNF release in the striatum, before, during, and following response learning.

The findings from Chapter 4 align with our hypothesis that physically active rats have an increased reliance on BDNF-TrkB signaling due to increased BDNF release.

There was no effect of cognitive priming on BDNF release; however, it is possible that there were methodological problems with this particular experiment, or it is possible that increased BDNF release is not the mechanism underlying cognitive activity-induced learning enhancements. In Chapter 5, to test our second hypothesis, GSK3 β inhibition, which is downstream of BDNF-TrkB signaling, was measured in the hippocampus and striatum of rats sacrificed in Chapter 3. GSK3 β inhibition in the hippocampus and striatum was increased following place and response training, but not following SA testing. Furthermore, blockade of BDNF-TrkB signaling had no effect on GSK3 β inhibition. Given that SA testing increases BDNF and that the place and response learning enhancements induced by SA testing were mediated by BDNF-TrkB signaling, it is likely that cognitive priming enhances learning via other signaling cascades downstream of BDNF-TrkB. Importantly, cognitive activity in the form of place and response training did increase GSK3 β inhibition in the hippocampus and striatum compared to untested naïve controls. Unfortunately, our naïve controls in Chapter 3/5 lacked cannula surgery and intracerebral infusions, thus it is possible that the GSK3 β inhibition observed in the place and response trained rats is due to surgery/infusions. However, this is unlikely given that the brain areas that lacked cannula implants and infusions in place and response trained rats also had increased GSK3 β inhibition.

Nonetheless, it is clear from the data in Chapter 5 that BDNF-TrkB signaling does not play a role in modulating GSK3 β inhibition. Thus, the inhibition of GSK3 β in place and response trained rats is being modulated via alternative signaling pathways. Although we were unable to test the effects of physical activity on GSK3 β inhibition in the hippocampus and striatum, others have found that treadmill exercise increases GSK3 β inhibition in the hippocampus compared to sedentary controls (Bayod et al., 2011; 2014). Reviewed in Figure 6.1, the findings of this dissertation point to increases in BDNF release and signaling in supporting the learning enhancements induced by cognitive and physical activities.

Cognitive and physical activity-induced neuroprotective adaptations in multiple memory systems: a mechanism for cognitive reserve?

Cognitive reserve, by definition, is maintained cognitive functioning in spite of neurodegenerative confrontation. Cognitive deficits associated with aging and neurodegenerative states (such as Alzheimer's disease (AD), Parkinson's disease (PD) and stroke reperfusion injury) often are a result of decreased BDNF signaling and GSK3 β hyperactivity (Tapia-Arancibia et al., 2008; Hooper et al., 2008; Lei et al., 2011; Collino et al., 2009; Ding et al., 2004b; Howells et al., 2000; Phillips et al., 1991; Croll et al., 1998; Ferrer et al., 1999). Furthermore, AD-, PD-, and ischemia-related neurodegeneration are ameliorated by increases in BDNF-TrkB signaling (Longo et al., 2007; Yoshimoto et al., 1995; Schabitz et al., 2004) and pharmacological inhibition of GSK3 β (Martinez et al., 2002; Morales-Garcia et al., 2013; Chuang et al., 2011; Hooper et al., 2008; Gimenez et al., 2012).

Both exercise and cognitive activity increase cognitive reserve and prevent or delay the onsets of cognitive deficits caused by aging and neurodegenerative disease (Wilson et al., 2007; Studenski et al., 2006; Chen et al., 2005; Ding et al., 2004a; 2004b). The findings in the current research show that cognitive and/or physical activities increase BDNF-TrkB signaling, BDNF release, and GSK3 β inhibition in the hippocampus and striatum (Chapters 2, 3, 4, and 5). Both the hippocampal and striatal memory systems have been implicated in neurodegenerative diseases such as AD, PD, and ischemic damage (Flock et al., 1998; Pulsinelli and Brierley, 1979). Thus, given the findings of the current research, engaging in cognitive and physical activities are practical, healthy, nonpharmacological interventions to boost cognitive reserve (see Figure 6.1) and to delay the molecular mechanisms underlying age-related cognitive decline and neurodegeneration.

FIGURES

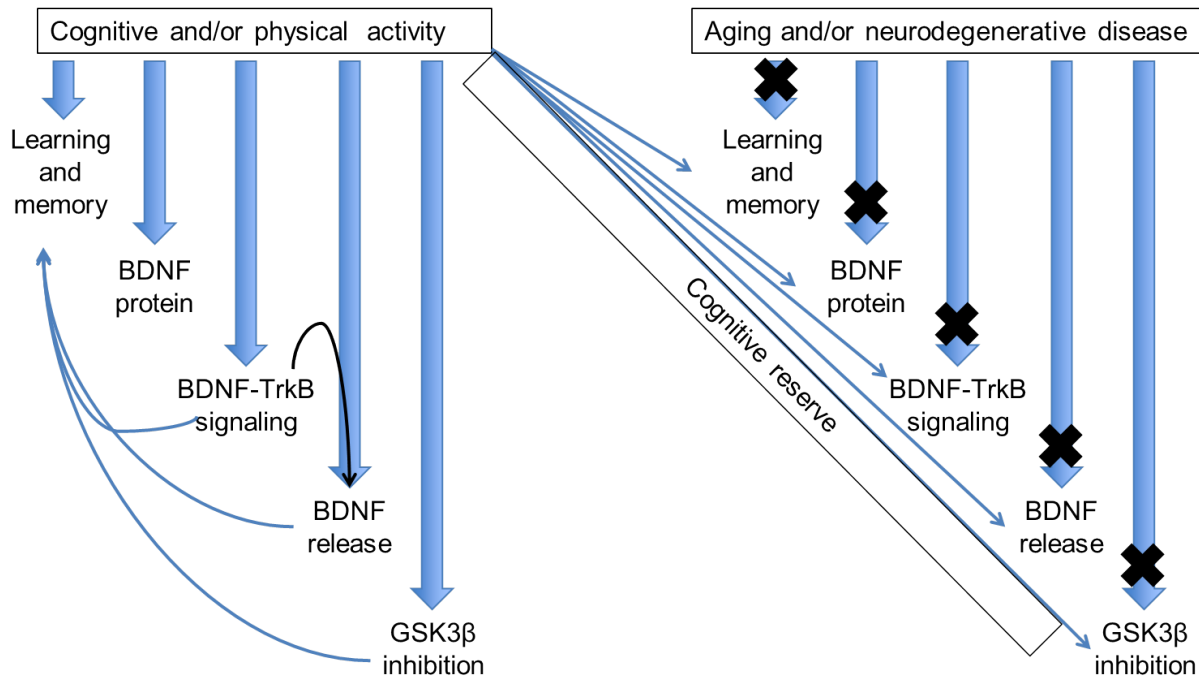


Figure 6.1. Mechanisms underlying “Use it and Boost it” promote cognitive reserve. Cognitive and/or physical activities enhance learning via BDNF-TrkB signaling, and possibly BDNF release in the hippocampus and striatum. Although BDNF-TrkB signaling induced by cognitive activity has no effect on GSK3β inhibition in the hippocampus and striatum, cognitive activity in the form of place and response learning leads to increases in GSK3β inhibition. In addition, long term exercise also increases GSK3β inhibition in the hippocampus. Others have shown that BDNF-TrkB signaling increases BDNF release. Given that individuals experiencing aging and neurodegenerative diseases have impairments in learning and memory, decreased BDNF-TrkB signaling, and increased GSK3β activity, engaging in regular cognitive and physical activity can possibly increase cognitive reserve so as to decrease cognitive decline.

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